University of Alberta

Cloning and Sequence Analysis of the *C. fetus* subsp. *fetus gyrA* Gene

by

Andrew Shiu-San Chau ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Medical Microbiology and Infectious Diseases

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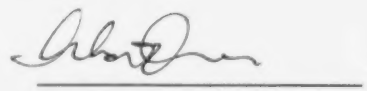

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Cloning and Sequence Analysis of *C. fetus* subsp. *fetus gyrA* submitted by Andrew Shiu-San Chau in partial fulfillment of the requirements for the degree of Master's of Science.

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For Joseph Chau, my father, my role model, my teacher and my friend.

Abstract

Campylobacter fetus subsp. *fetus* are the causative agent of sporadic abortions in sheep and cattle and are ocassionally associated with systemic infections in humans. Unlike the well known pathogen *C. jejuni, C. fetus* has a high intrinsic resistance to the quinolone antibiotic nalidixic acid (256 μg/ml). In other bacteria that have been studied such as *Escherichia coli* or *Staphlococcus aureus*, high levels of quinolone resistance has been associated with single amino acid substitutions in the GyrA subunit of DNA gyrase. These mutations have been localized to a region of GyrA known as the quinlone resistance determining region, or QRDR, which spans amino acids 67 to 106 in the *E. coli* gyrase. This region of the enzyme has now been implicated in fluoroquinolone resistance in a number of different bacteria. Results from *C. fetus* UA60 *gyrA* sequencing indicate that the high level nalidixic acid resistance obesrved in this species is not associated with the QRDR. This region, spanning amino acid residues 71 to 110 in the *C. fetus* GyrA, was highly sequence conserved when compared to the analogous region from a nalidixic acid sensitive *C. jejuni* UA580. The *C. fetus* protein had high overall identity with the *C. jejuni* enzyme as well as the *Helicobacter pylori* sequence. There was about an overall identity of about 50% with other GyrA proteins. Examination of a fluroquinolone resistant *C. fetus* subsp. *fetus* isolate as well as a laboratory mutant of UA60 selected for ciprofloxacin resistance showed that this specific resistance is associated with the QRDR. Both strains had identical Tyr substitutions at Asp-91, which is equivalent to the Asp-87 mutations in *E. coli* and Asp-90 in *C. jejuni* previously described as being associated with fluroquinolone resistance. These observations strongly suggest there are most likely at least two different resistance mechanisms active against quinolone antibiotics in *C. fetus* subsp. *fetus*. One is

similar to the classical resistance illustrated by the association with single amino acid substitutions in the QRDR and the other is due to an, as yet, uncharacterized mechanism that is specific for nalidixic acid and does not appear to involve the QRDR.

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List of Abbreviations

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHI	Brain Heart Infusion
Blast	Basic Local Alignment Search Tool)
bp	basepair
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CFU	colony forming units
dNTP	deoxynucleoside triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuracil triphosphate
ddATP	dideoxyadenosine triphophate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DIG	dioxygenin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
G + C	guanosine + cytosine
GCG	Wisconsin Genetics Computer Group
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kDA	kilodaltons
LB	Luria Bertani
LMP	low melting point
MWCO	molecular weight cut-off
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
MOPS	4-morpholinepropanesulfonic acid
NaOAc	sodium acetate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	1,4-piperazinebis(ethanesulfonic acid)
QRDR	quinolone resistance determining region
Rnase	ribonuclease
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIC	supercoiling inhibiting concentration
SSC	standard sodium citrate
T_m	melting temperature
Tris	tris (hydroxymethyl) aminomethane

Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
U	unit
UV	ultraviolet
xg	relative centrifugal force
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. Introduction

1.1 General Characteristics of Campylobacters

Campylobacter spp. were originally classified with vibrios such as *Vibrio cholerae* in the family *Vibrionaceae* (McFaydean and Stockman, 1913), however, the genus *Campylobacter*, derived from the Greek campylo for curved, and bacter for rod, was created to differentiate the campylobacters from the vibrios because of their many dissimilarities (Sebald and Véron, 1963; Véron and Chatelain, 1973). *Campylobacter* spp. are small (0.5 to 8 μm long and 0.2 to 0.5 μm wide), nonsporeforming, Gram-negative rods with a curved, spiral, or S-shaped morphology (Sebald and Véron, 1963). However, *C. jejuni* has been shown to have a pleiomorphic morphology *in vitro* that correlates with the age of the culture (Ng *et al.*, 1985; Griffiths, 1993). On agar, young organisms in cultures were spiral or S-shaped whereas organisms from older cultures tended to be more coccoid. In liquid cultures older cells were observed to be two to four times the length of log phase cells.

Campylobacters generally have a single polar unsheathed flagellum at one or both ends and a characteristic corkscrewlike darting motility seen under phase-contrast microscopy (Smibert, 1978). They have a G + C content ranging between 28 - 38 mol%. Most are microaerophilic (5 - 10% O_2 and 3 - 10% CO_2) but some are oxygen-tolerant or require anaerobic conditions for growth. Optimal growth temperatures range from 25^0C to 42^0C, with different species exhibiting different temperature optima (reviewed in Penner, 1988). Campylobacters possess a respiratory type of metabolism but neither oxidize nor ferment carbohydrates, instead, they use energy sources such as amino acids or

tricarboxylic acid cycle intermediates. There is 13 diverse species within the genus *Campylobacter* are 13 diverse species and subspecies (Penner, 1988). *C. jejuni* is the most significant human pathogen followed by *C. coli* and then *C. fetus*. *C. jejuni* are responsible for the majority of human bacterial gastrointestinal diseases. Differentiation between species is made on the basis of G + C content, sites of infection, temperature requirements for growth, nalidixic acid and cephalothin resistance profiles, serum resistance, and biochemical tests such as the catalase reaction, hippurate hydrolysis or H_2S production.

1.2 History of *Campylobacter fetus*

C. fetus is the type species of the genus and is subdivided into two subspecies: *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Both subspecies cross-react in DNA hybridization tests (Harvey and Greenwood, 1983; Roop *et al.*, 1984), have similar G + C content (Véron and Chatelaine, 1973; Smibert, 1984), and agglutinate with antiserum against the same serotype. Their similarities in these aspects had prompted some to argue against the delineation between subspecies. The species *C. fetus* is the same as '*Vibrio fetus*' first described by Smith and Taylor (1919). Since then, Florent (1959) recognized that two distinct diseases could be attributed to two varieties of '*V. fetus*' which he named '*V. fetus*' var. intestinalis and '*V. fetus*' var. venerealis. These classifications correspond to what are currently recognized as *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* respectively. The former is found in bovine and ovine intestines and causes sporadic abortions in cattle and sheep (Florent, 1959). The latter is isolated from the prepuce of carrier bulls and is the causative agent of bovine genital campylobacteriosis, a sexually transmitted disease that causes sterility in cows (Samuelson and Winter, 1966). *C. fetus* subsp. *fetus* have a broad host range; in addition to causing abortions in cattle and sheep,

they also infect horses, poultry, reptiles, and humans (Blaser, 1993). Vinzent (1947) reported the first case of *C. fetus* as a human pathogen in an abortion caused by this organism.

1.3 *C. fetus* epidemiology

Unlike *C. jejuni*, *C. fetus* is an infrequent cause of enteric disease in healthy humans (Allerberger *et al.*, 1991; Farrugia *et al.*, 1994; Grollier *et al.*, 1993) which explains the paucity of fecal isolates. In humans, *C. fetus* is most often cultured from the blood of infected individuals, which highlights the potential seriousness of the diseases associated with infection (Penner, 1988). Diarrhea caused by *C. jejuni* or *C. coli* are self-limiting and rarely require antibiotic therapy, whereas *C. fetus* infections, because of their systemic nature need to be treated. The most frequent disease symptoms are septicemias in those with predisposing factors, such as immunocompromised individuals (Gurrent *et al.*, 1978; Rettig, 1979; Skirrow *et al.*, 1993; Ullmann *et al.*, 1982; Wyatt *et al.*, 1977). There have also been reports of meningitis, pericarditis, peritonitis, salpingitis, septic arthritis, and abscesses (Penner, 1988). In contrast, there have been no reports of *C. fetus* subsp. *venerealis* infection in humans. Early studies of *Campylobacter* bacteremia indicated a majority of blood isolates were *C. fetus* (Guerrant *et al.*, 1978) as they outnumbered *C. jejuni* and *C. coli* five to one. By contrast, a survey of enteric cases from 1981-1991 by Skirrow and colleagues (1993) showed *C. jejuni* and *C. coli* accounted for 89% of the 394 enteric cases reported. This is supported by the reports of Lastovica and Penner (1983), Spelman *et al.* (1986), and Tauxe *et al.* (1988) in which the bulk of Campylobacters identified from non-spepticemic cases were either *C. jejuni* or *C. coli*. These data may be biased, as clinical isolations usually capitalize on the thermophilic nature of the enteric

Campylobacter species by incubating cultures at 42^{0}C and in the presence of cephalothin (Penner, 1988; Harvey and Greenwood, 1983). Both these conditions would be selective against *C. fetus* but favor isolation of *C. jejuni* or *C. coli*. There may also be geographic differences since the two studies that had the most similar results were both conducted in the United States, whereas the other studies were from Europe, Africa and Australia.

1.4 *C. fetus* biochemical and virulence characteristics

The inability of *C. fetus* to grow at 42^{0}C as well as its sensitivity to cephalothin are useful in differentiating the organisms from other Campylobacters (Penner, 1989). They are also catalase positive, hippurate negative, and do not produce H_2S in the triple sugar iron agar test. Another differentiating feature is that *C. fetus* are naturally resistant to nalidixic acid to high levels but there is no cross-resistance to fluoroquinolones such as norfloxacin or ciprofloxacin. Unlike *C. jejuni* and *C. coli*, *C. fetus* do not appear to become coccoid as cultures age. Amano and Shibata (1992) found that peptidoglycan was degraded in both *C. jejuni* and *C. coli* as organisms aged but not in *C. fetus*. They speculated that morphological differences were related to this differential degredation. *C. fetus* are also serum resistant, whereas *C. jejuni* and *C. coli* are not, and this is probably due to the presence of an extensive surface protein (S) layer (Blaser *et al.*, 1987; Blaser *et al.*, 1988; Dubreuil *et al.*, 1988; McCoy *et al.*, 1975) that protects the organims from complement-mediated lysis. The S layer is a two-dimensional protein lattice that represents the outermost surface in many bacteria (Koval and Murray, 1984; Sleytr and Messner, 1988; Smit, 1986). In *C. fetus*, in addition to serum resistance, the presence of this S layer confers protection from phagocytosis and is probably responsible for inhibiting the binding of the C3b component of complement to the outer surface. This crystalline structure is composed

of proteins with M_r ranging from 98,000 to 149,000 (Dubreuil *et al.*, 1988; McCoy *et al.*, 1976; Myers, 1971; Pei *et al*, 1988) and western blot data suggests that differential expression of the predominant S layer subunit protein may be responsible for antigenic variation in *C. fetus* (Dubreuil *et al.*, 1990). Shurig *et al.* (1975; 1978) demonstrated that *C. fetus* can undergo antigenic changes *in vivo* and this may represent a mechanism for maintenance in asymptomatic carriers (Corbeil *et al.*, 1975). There is a high degree of probability that differential S-layer protein expression is responsible since the O antigen of *C. fetus* is not differentially expressed (Corbeil *et al.*, 1975; Shurig *et al.*, 1978). Dubreuil *et al.* (1990) speculate this may be a defense strategy on the part of *C. fetus* for evading humoral or immune defense factors, which fits well with the vascular or CNS-associated nature of *C. fetus* pathogenesis.

1.5 Introduction to DNA gyrase

DNA gyrase is a member of the family of enzymes known as toposisomerases. As their name would imply, they are proteins that modify the topological structure of DNA. Both prokaryotic and eukaryotic cells possess topoisomerases but gyrases appear to be unique to bacteria. Of the four different classes of topoisomerases, DNA gyrase is a member of the type II enzymes (Gellert *et al.*, 1976a) and is the only known enzyme that can introduce negative supercoils into DNA. This process occurs in steps of two, which means the linking number changes by two with the introduction of each negative supercoil. The mechanistic interpretation of this observation is a transient double-stranded DNA breakage and passage of one strand through the duplex (Mizuuchi *et al.*, 1980; Brown and Cozzarelli, 1979). Type I topoisomerases, in contrast, catalyze DNA interconversions in steps of one. In addition, the first topoisomerase to be described,

prokaryotic topoisomerase I, relaxes negatively supercoiled DNA. The assumption is that both DNA gyrase and topoisomerase I act in a coordinated fashion to modify the level of supercoiling within a cell and that any alteration in the function of either enzyme would result in a concomitant perturbation in the degree of supercoiling. Other functions of DNA gyrase are the relaxation of supercoiled DNA (Higgins et al., 1978; Gellert et al., 1979), catenating and decatenating of two double stranded DNA circles (Kreuzer and Cozzarelli, 1980), and unknotting of single stranded DNA. In order to perform its functions, DNA gyrase must bind DNA, cleave the DNA, pass a strand through the duplex, reunite the strands and hydrolyze ATP.

1.6 Physical characteristics of DNA gyrase

DNA gyrase is hypothesized to have the structure A_2B_2 as inferred from studies that determine the molar ratio of both A and B subunits in a gyrase-DNA complex (Sugino *et al.*, 1980). Furthermore, sedimentation analysis of gyrase complexed with DNA fragments of known molecular size suggested a molecular weight of 470 kDa, the same as that calculated for a molecule with an A_2B_2 structure (Klevan and Wang, 1980). The nucleic acid sequencing data from the first *gyrA* gene to be characterized, the *E. coli* gyrase, showed the A subunit to be 876 amino acids in length with an estimated M_r of 97 kDa (Swanberg and Wang, 1987). The B subunit is 804 amino acids in length and has a size of 90 kDa (Yamagishi *et al.*, 1986). Most gyrases that have been sequenced or studied have subunit sizes similar to *E. coli* (reviewed in Reece and Maxwell, 1993), ranging from 90 to 115 kDa for GyrA and 71 to 96 kDa for GyrB. The function of the A subunit appears to be in DNA breakage and reunion in addition to DNA binding (Sugino *et al.*, 1977), whereas

the B subunit has an ATPase function (Sugino *et al.*, 1978; Mizuuchi *et al.*, 1978) which requires both the A subunit and DNA for full activity.

1.7 Theoretical description of DNA supercoiling

Normally, closed circular DNA molecules extracted from bacterial cells exhibit a linking deficit as compared to a nicked or linear DNA (Drlica, 1992). This means there are fewer duplex turns than would be expected and this deficit is commonly referred to as negative supercoiling. The inverse would be true of positive supercoiling. This property of a physically intact circular DNA molecule can be described by a series of simple equations. The concept of linking number can be described by the relationship (White, 1976; Fuller, 1971; Crick, 1976):

$$Lk = Tw + Wr$$

Lk, the linking number, is an integer and is invariant as long as two strands remain intact (ie., The phosphodiester backbone is continuous.) describes the number of times two strands of a DNA circle are interwound. This is a measure of the number of right-handed versus left-handed crossings in a planar duplex circle. If this value is positive, this means there are more right-handed crossings than left-handed crossings which is generally the case since DNA is a right-handed helix . Tw is the twist, which is equivalent to the number of turns of the double helix - this is equivalent to the pitch. Wr is the writhe and is a measure of the contortion of the helix axis in space or, in other words, the representation of the concept of supercoiling as the spiraling of the longitudinal axis of the helix. If nicked DNA is closed by DNA ligase, this relaxed molecule will have linking numbers

approximately equal to the number of duplex turns (Tw^o). This value is equal to the number of base pairs divided by the pitch (Rhodes and Klug, 1980; Peck and Wang, 1981; Shore and Baldwin, 1983; Horowitz and Wang, 1984), or:

$$Tw^o = N/10.5$$

In reality, on an agarose gel, these DNA molecules would appear as a ladder since a series of DNA topoisomers is formed with the linking number differing by one from each topoisomer to the next (Depew and Wang, 1975; Pulleyblank *et al.*, 1975; Keller and Wendel, 1974). The distribution of topoisomers is approximately Gaussian with the value of the most abundant species Lk^o approximating Tw^o. The concept of supercoiling can then be illustrated by the equation (Crick *et al.*, 1979):

$$\Delta Lk = Lk - Lk^o$$

If ΔLk is negative, the DNA is said to have a linking deficit, thus, will be underwound or negatively supercoiled. Alternatively, if ΔLk is positive, the DNA is positively supercoiled. However, the linking number determined in this manner assumes the axis of the DNA molecule lies in a plane. In a situation where the DNA is wrapped around a protein, such as in a biological environment, the axis is no longer planar, thus the linking number is described by (White *et al.*, 1988):

$$Lk = SLk + \Phi$$

SLk is the surface linking number which accounts for the perturbations in twist and writhe due to the surface configuration of the protein the duplex circle is wrapped around. Φ is the winding number and is a function of the pitch relative to the surface on which the DNA axis lies. In real terms, because the linking number of a closed circular DNA duplex cannot change, the presence of intercalating dyes such as ethidium bromide will alter the twist and writhe to accommodate the torsional strain. However, a nicked duplex DNA can change either its twist or writhe to compensate for the perturbation in DNA structure. The only method by which duplex DNA circles can change their linking numbers is by a physical breakage and reunion of the phosphodiester backbone (Depew and Wang, 1975; Pulleyblank *et al.*, 1975). Relieving the torsional strain introduced into DNA via the breaking and resealing mechanism is one of the functions of DNA gyrase and is probably the only way that bacteria are able to reduce the free energy of the nucleoid material while still maintaining a biologically active molecule. The implication that Lk is related to the state of supercoiling and that Lk can only be altered by a physical breakage of DNA supports this idea. This can be illustrated in the example of DNA replication, where DNA is locally unwound at the replication fork facilitating synthesis of a daughter strand. Because duplex DNA is a right-handed helix and since there is not a physical breakage of either strand, this unwinding may introduce torsional strain on the molecule. Introducing negative supercoils may have a two-fold effect: this stress can be relieved allowing the DNA molecule to maintain a energetically favorable conformation; and the negative supercoiling may also enhance unwinding of the DNA at the replication fork.

1.8 Regulation of DNA gyrase expression and supercoiling

Expression of DNA gyrase is controlled by the extent that DNA is supercoiled within the cell (Menzel and Gellert, 1983). There are three general categories of factors contributing to supercoiling levels within a cell. These are those which: (i) affect DNA topoisomerases; (ii) alter DNA structure; or (iii) influence cellular energetics (Drlica, 1992). *In vivo*, the presence of gyrase inhibitors caused up to a ten-fold increase in the expression of both *gyrA* and *gyrB* (Menzel and Gellert, 1983). Presumably, there is a reduction in DNA supercoiling, which stimulates gyrase synthesis. Intercalating agents such as ethidium bromide will also affect the degree of supercoiling. Unless the DNA strand is physically broken to accommodate the torsional strain induced by the dye, the only way by which closed circular DNA can respond is to alter both the writhe and the twist. This means both the pitch and the spiraling of the axis are affected. As a result, these chemicals act to reduce the apparent level of supercoiling. Those factors which alter cellular energetics, which in turn affect ATP levels, will also affect the level of supercoiling because it is an ATP-dependent reaction. As [ATP]/[ADP] levels drop, there is an increase in relaxation. As [ATP]/[ADP] levels rise, so does the amount of supercoiling.

1.9 Functions of DNA gyrase

The different activities associated with DNA gyrase require both the A and B subunits. The introduction of supercoils, relaxation of negative and positive supercoils, catenation and decatenation of DNA circles, resolution of topologically knotted DNA and DNA cleavage all require both subunits despite these functions being associated with the GyrA. The reason for this is that, except for the cleavage reaction and relaxation of

negative supercoils, these activities also have a requirement for ATP. Those activities that can proceed in the absence of ATP but still require both subunits may have a requirement for the holoenzyme structure. These functions, for the most part, have only been described *in vitro* with *in vivo* activities inferred from these results. The degree of supercoiling is thought to have influence on *in vivo* functions such as transcription, translation and replication. There is evidence that the level of supercoiling may contribute to suppression of gene transcription. Conversely, negatively supercoiled DNA may be more easily unwound, facilitating RNA polymerase binding, hence, promoting transcription of some genes. The precise function of DNA gyrase in replication is not clear, however, it is known that gyrase inhibitors also prevent replication and temperature sensitive gyrase mutants are replication deficient at non-permissive temperatures (Kreuzer and Cozzarelli, 1979; Orr and Staudenbauer, 1981). A related function is involvement in the decatenation and partitioning of daughter chromosomes during cell division (Hiraga, 1990; Schaechter, 1990). GyrB temperature-sensitive mutants yielded chromosomal doublets at non-permissive temperatures which could be resolved by gyrase *in vitro* (Steck and Drlica, 1984). Although the significance of a GyrB mutation causing this phenotype is not known, it does demonstrate the involvement of gyrase in these functions. At a more basic level, the extent of supercoiling also affects the overall volume that a DNA molecule occupies and may aid in packaging the nucleic acid molecule in a cell.

1.10 Antibiotic resistance mechanisms in Campylobacters

Antibiotic resistance in *Campylobacter* spp. has been studied by a number of investigators (Taylor and Courvalin, 1988; Taylor, 1992b). *C. jejuni*, *C. coli*, and other closely related Campylobacters were found to be intrinsically resistant to a wide range of

antibiotics, including bacitracin (MIC, ≥512 μg/ml), cephalothin (MIC, 64-≥512 μg/ml), novobiocin (MIC, 64-≥512 μg/ml), rifampin(MIC, 8-≥128 μg/ml), streptogramin B (MIC, ≥256 μg/ml), trimethoprim (MIC, 256-≥512 μg/ml), and vancomycin (MIC, 128-≥512 μg/ml). These resisitance mechanisms have not been characterized but are thought to result from decreased permeability of the outer membrane. These resistances are in contrast to acquired characters, such as those that arise via chromosomal mutations, plasmids, or transposons. In campylobacters, resistances have been described against quinolones and fluoroquinolones, β-lactams, aminoglycosides, macrolides and lincosamides, and tetracycline.

1.11 Quinolone and fluoroquinolone resistance in Campylobacters

Compared to other bacteria, campylobacters exhibit an increased intrinsic resistance to quinolone antibiotics in general (Taylor *et al.*, 1985; Hooper and Wolfson, 1993) and highly resistant strains are selectable in a single-step mutation unlike other Gram negative enteric pathogens (Gootz and Martin, 1991). *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. lari*, and *C. hyointestinalis* are all intrinsically resistant to nalidixic acid to high concentrations (128 to 256 μg/ml). Despite this, *C. fetus* subspp. and *C. hyointestinalis* are fluoroquinolone sensitive, but *C. lari* is also cross-resistant to both ciprofloxacin and enoxacin (Taylor and Courvalin, 1988). Spontaneous *C. jejuni* and *C. coli* mutants resistant to nalidixic acid are also cross-resistant to fluoroquinolones. These differences among *Campylobacter* spp. would indicate there are at least two mechanisms of quinolone resistance. One mechanism would be responsible for the nalidixic acid resistance profile of *C. fetus* and the other, presumably distinct, mechanism would confer

both quinolone and fluoroquinolone resistance (Taylor and Courvalin, 1988). Wang and Taylor (1993) found that laboratory mutants of *C. jejuni* UA580 resistant to nalidixic acid shared mutations in the quinolone resistance determining region (QRDR) of GyrA with other bacteria. Consistent with previous findings, these mutants were also resistant to ciprofloxacin.

1.12 Tetracycline resistance in Campylobacters

Resistance to tetracycline in both *C. jejuni* and *C. coli* has been shown to be plasmid mediated (Taylor et al, 1980). These plasmids are self-transmissible with limited host range; they will transfer to other Campylobacters such as *C. fetus* subsp. *fetus* but not to any members of the *Enterobactericieae*. The resistance determinant, *tetO*, is highly homologous to *tetM* from *Enterococcus pnuemoniae*. From nucleic acid sequence data, *in vitro* transcription-translation studies, and *E. coli* minicell and maxicell analysis, the *tetO* coding sequence is 1,911 nucleotides in length encoding for a protein of 637 amino acids with a M_r of 68,000-72,000 Da (Manavathu *et al.*, 1988; Sougakoff *et al.*, 1987; Taylor *et al.*, 1987). Manavathu *et al.* (1990) found that TetO activity, similarly to TetM, was at the level of protein synthesis by measuring the amount of [^{35}S]methionine incorporation in *E. coli* JM107 in the presence and absence of a *tetO* plasmid. Without TetO, tetracycline was found to inhibit [^{35}S] incorporation but four times as much tetracycline was required to achieve the same effect in the presence of a *tetO* plasmid. Similar results were observed with in vitro poly(U)-directed polyphenyalanine incorporation studies (Manavathu et al., 1990). However, there was no inhibition of tetracycline binding of ribosomes. It is generally thought that tetracycline binds to a single high-affinity site within the 30S subunit of the 70S ribosomal subunit, thus preventing entry of the aminoacyl-tRNA at the A site

(Goldman *et al.*, 1983). This would effectively inhibit elongation of the nascent amino acid chain. The mechanism of resistance has not been elucidated but it is suspected that TetO interacts with ribosomes. TetO was found to be homologous to the N-terminal regions of elongation factors Tu (EF-Tu) and G (EF-G) which are associated with ribosomes (Manavathu *et al.*, 1990). They all share common structural motifs with GTP-binding proteins and GTPases.

1.13 Chloramphenicol resistance in Campylobacters

The incidence of chloramphenicol resistance in *Campylobacter* isolates is considerably lower than that of tetracycline. A Centers for Disease Control survey from 1987 to 1989 found that 43% of strains isolated from humans in the United States were tetracycline resistant to levels >16 μg/ml. In comparison, there were no isolates resistant to greater than 8 μg/ml chloramphenicol (Tenover *et al.*, 1992). Sagara *et al.* (1987) identified and cloned a high level chloramphenicol resistance determinant from *C. coli.* Nucleotide sequence analysis by Wang and Taylor (1990) revealed this gene, with an ORF of 621 bp encoding a protein of 207 amino acids with a deduced M_r of 24,294, to be homologous to other chloramphenicol acetytransferases (*cat*). The mechanism of CAT-mediated resistance is well known and studied. The enzyme catalyzes the inactivation of the antibiotic via an acetyl-S-CoA-dependent acetylation of the 3-hydroxyl group of chloramphenicol. The 3-acetoxy chloramphenicol product is unable to bind ribosomes (Shaw, 1983). The *C. coli* determinant presumably has the same mechanism since there is a highly conserved region in the C-terminus of the protein corresponding to the active site.

1.14 Macrolide resistance in Campylobacters

Although <3% of clinical isolates are resistant to macrolides, they tend to exhibit extremely high MICs (>1,024 μg/ml) and usually display cross-resistance to spiramycin, lincomycin, clindamycin and azithromycin to high levels (Taylor, 1992). Unlike either tetracycline or chloramphenicol, erythromycin resistance appears to be chromosomally mediated as the phenotype is independent of the presence of plasmids. In addition, natural transformation studies (Yan and Taylor, 1991) showed that a chromosomal fragment of an Ery^r *C. coli* strain could be taken up and recombine with the chromosome of Ery^s strains conferring erythromycin resistance. This type of resistance is associated more with *C. coli* than *C. jejuni* and has not been studied in other Campylobacters. The mechanism of this resistance is not known nor has the chromosomal locus been identified. It is known that this resistance is constitutive and not inducible, does not involve an RNA methylase, is not due to enzymes capable of modifying erythromycin and does not result from an efflux mechanism. It is known that this resistance is at the protein synthesis level and that resistant strains bind erythromycin with less affinity than sensitive strains (Yan and Taylor, 1991).

1.15 Aminoglycoside resistance in Campylobacters

Kanamycin resistance in *C. coli* was first described in by Lambert et al. (1985) and seems to be more associated with this species than with *C. jejuni*. Trieu-Cuot and co-workers (1985) cloned and sequenced the kanamycin resistance determinant from *C. coli* plasmid pIP1433. This gene was 1427 bp in size and encoded for *aphA-3*, a type III 3'-

aminolycoside phosphotransferase. Because this gene had been previously found in gram-positive cocci, it was thought this determinant was acquired from a gram-positive coccus.

1.16 History of quinolone antibiotics

The discovery of quinolones as antibiotic compounds was serendipitous. Lesher and co-workers (1962) accidentally discovered that 7-chloro-1-ethyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, a byproduct in the synthesis of chloroquine, an anti-malarial drug, had antibacterial activity. This class of drugs has a basic two ring structure and congeners differ in their composition of substituent groups at positions N1, C6, or C7. In 1963, nalidixic acid was introduced as the first quinolone for clinical use (S. Rádl, 1990). This was quickly followed by other more effective quinolones. These, along with the coumarin drugs, were found to be active against DNA gyrase (Gellert *et al.*, 1977; Sugino *et al.*, 1977; and Gellert *et al.*, 1976; Hooper *et al.*, 1982; Smith, 1986; Sugino *et al.*, 1978). Quinolones have activity against both the A and B subunits of gyrase, whereas the coumarin drugs inhibit only the B subunit. In addition to nalidixic acid, examples of quinolones include oxolinic acid, enoxacin, ciprofloxacin, norfloxacin and ofloxacin. The latter three are considered fluoroquinolones due to the presence of the fluorine at position 6 (refer to Figure 1.1). The 'classical' quinolones possess N1 ethyl groups or other groups of similar size. Newer quinolones have cyclopropyl or fluorophenyl groups substituted in N1 which seemed to afford the same potency but broader spectrum of activity. Subsequent to this, analogs with a fluorine at position 6, coupled with a piperazine ring substituent at position 7, exhibited the greatest antibacterial activities with the broadest spectra (Rádl, 1990). These drugs are attractive because of the uniqueness of DNA gyrase to prokaryotes, which reduces the potential for toxicity to humans upon therapeutic

administration. Ease of absorption by and distribution throughout the body, and a wide spectrum of activity against both gram positive and gram negative bacteria are the attractive features of this class of drugs.

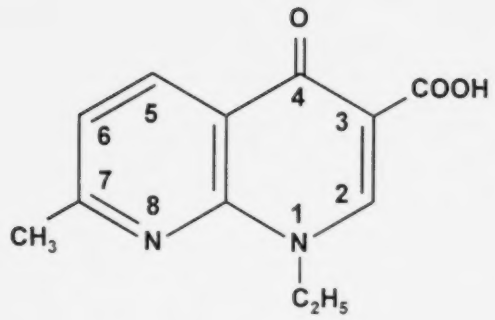
1.17 Quinolone targets and action

Along with the experimental evidence that DNA gyrase is the intracellular target for quinolones, there is evidence of other, more generalized effects on cell metabolism and growth. These include increased damage to DNA; inhibition of DNA synthesis and nucleoid formation, which indicates a loss of chromosome decatenation subsequent to DNA synthesis; inhibition of RNA and protein synthesis at high quinolone concentrations; and cell filamentation (reviewed in Hooper and Wolfson, 1993). The mechanism by which quinolones kill bacteria is not known, but from experimental data, two alternate mechanisms have been proposed, described simply as mechanisms A and B. This reflects the finding that under certain biological conditions some quinolones lose their activity, whereas others do not. For example, rifampicin or chloramphenicol treatment yields loss of bactericidal activity of norfloxacin and nalidixic acid but not ciprofloxacin, ofloxacin, lomefloxacin, perfloxacin nor fleroxacin (Lewin *et al.*, 1989; Lewin *et al.*, 1990). One model proposes an initial association of quinolones with gyrase-DNA complex leading to damage of DNA caused by strand breakage. This damage is then nonrepairable. This is consistent with the findings that quinolones promote DNA gyrase-mediated DNA cleavage, induce the SOS system and have greater activity against SOS-deficient strains (Hooper and Wolfson, 1993).

Figure 1.1. Structure of quinolone antibiotic compounds.

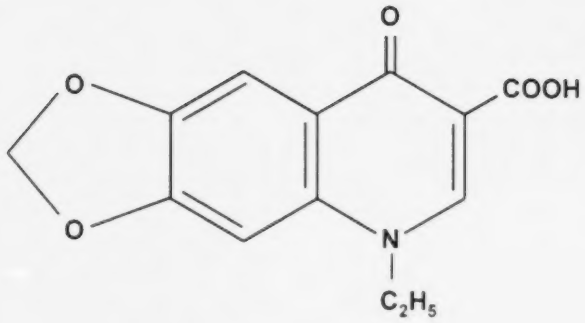
(A) Nalidixic acid; (B) oxolinic acid; and (C) ciproflocaxin. The numbering scheme for the ring members is indicated in nalidixic acid.example (A).

A.



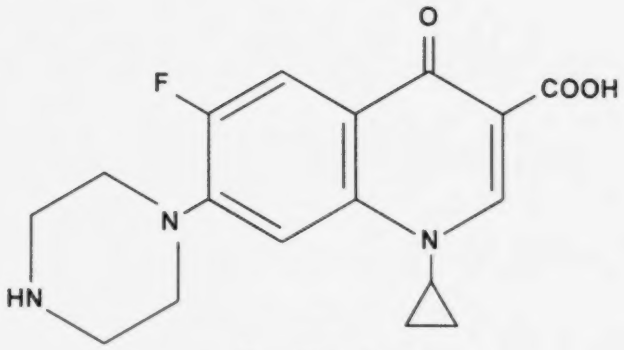
Nalidixic Acid

B.



Oxolinic Acid

C.



Ciprofloxacin

1.18 Quinolone resistance associated with amino acid substitutions in the QRDR

There has been an increase in the clinical incidence of quinolone resistance among prokaryotes. Resistance to nalidixic acid was first reported in 1966 during the treatment of urinary tract infections (Ronald *et al.*, 1966). This resistance was localized to the DNA gyrase (Gellert *at al.*, 1977) in *E. coli* on the basis of genetic and biochemical data. Since then, purified mutant DNA gyrase subunit complementation studies in *B. subtilis* (Sugino and Bott, 1980), *E. faecalis* (Nakanishi *et al.*, 1991), *S. aureus* (Okuda *et al.*, 1991), *C. jejuni* (Gootz and Martin, 1991), *S. marcescens* (Masecar and Robillard, 1991), *P. aeruginosa* (Inoue *et al.*, 1987; Masecar *et al.*, 1990; Miller and Scurlock, 1983; Robillard and Scarpa, 1988), *H. influenzae* (Setlow et al., 1985), and *C. freundii* (Aoyama *et al.*, 1988) have confirmed that GyrA is an intracellular target for quinolone antibiotics. The majority of quinolone-resistant gyrase mutants have mapped to a small region near the N-terminus of the GyrA subunit (Aoyama *et al.*, 1988; Hooper *et al.*, 1982; Cullen *et al.*, 1989; Yoshida *et al.*, 1988) from amino acid residues equivalent to 67 through 106 in *E. coli* KL16 (Yoshida *et al.*, 1990). This stretch has been called the quinolone resistance determining region (QRDR) (Yoshida *et al.*, 1990). The majority of those mutations conferring higher levels of resistance map to *E. coli* residue 83, which is a serine in the wild-type enzyme (Yoshida *et al.*, 1988; Yoshida *et al.*, 1990; Oram *et al.*, 1991; Cullen *et al.*, 1989). This same mutation is also seen in other bacteria such as *Campylobacter jejuni* (Wang *et al.*, 1992), *Helicobacter pylori* (Moore *et al.*, 1995), *Haemophilus influenzae* (Setlow *et al.*, 1985), *Staphylococcus aureus* (Fasching *et al.*, 1991; Goswitz *et al.*, 1992; Sreedharan *et al.*, 1990), and *Psuedomonas aeruginosa* (Kureishi *et al.*, 1994) whose mutant gryases have been sequenced to date.

The greatest increases in resistance are observed with substitutions of Ser for large, hydrophobic residues such as Leu or Trp in *E. coli* (Yosida *et al.*, 1988) or Ile in *C. jejuni* (Wang *et al.*, 1992). Substitutions with less bulky, nonpolar amino acids, such as Ala, are associated with smaller increases in MICs. The specifics of this resistance are not well understood but it has been hypothesized that a mutation in GyrA alters the affinity of quinolones for the DNA gyrase-DNA complex (Hooper and Wolfson, 1993), and both the polarity and size of side groups play a role. Alternatively, mutations in DNA gyrase may block access of quinolones to single-stranded-DNA pockets created by DNA gyrase (Yoshida *et al.*, 1990) or decrease quinolone binding to the pocket without affecting drug-protein interactions (Hooper and Wolfson, 1993). In *E. coli*, it has been shown that a DNA-gyrase complex with a mutation in GyrA (Trp-83) binds norfloxacin with less affinity than wild-type enzymes (Willmott and Maxwell, 1993). However, in the absence of DNA, the amount of binding was equally low in both wild-type and mutant. Mutations in the QRDR may be spatially oriented near Tyr-122, which binds each 5'-phosphate end of transiently cleaved duplex DNA (Yoshida *et al.*, 1990). Horowitz and Wang (1987) found that oxolinic acid inhibits the reunion of these DNA ends. There have also been mutations conferring moderate increases in quinolone resistance mapping near the middle of GyrB (Yagamashi *et al.*, 1986) although this mechanism is not understood.

1.19 Experimental evidence for quinolone action

In addition to *in vivo* evidence, it can be demonstrated that DNA gyrase is a target for quinolone drugs with *in vitro* supercoiling assays. Purified enzyme, in the presence of Mg^{2+}, ATP and spermidine, can supercoil relaxed, closed-circular plasmid ColE1 DNA (Staudenbauer and Orr, 1981). This is visualized on an agarose gel as an increase in

electrophoretic mobility as compared to the relaxed species. However, in the presence of quinolone antibiotics, there is a lessening of supercoiling activity, eventually becoming a total cessation at that drug's MIC for gyrase (Gootz and Martin, 1991). In addition, it has been shown with [^{3}H]enoxacin binding studies that quinolones bind to gyrase-DNA complexes and not to either gyrase nor duplex DNA alone (Shen et al., 19.

1.20 Model of quinolone-DNA interaction

There are a number of observations that have contributed to the development of a cooperative binding model for quinolone inhibition of DNA gyrase. Binding studies with radioactively labeled nalidixic acid found that it did not bind to double stranded DNA (Bourguignon *et al.*, 1973). In fact, there was evidence that nalidixic acid bound to single stranded DNA substrates in the presence of excess copper ions (Crumplin *et al.*, 1980; Dreyfuss and Midglely, 1983). Subsequent studies with radioactively labeled norfloxacin by Shen and his co-workers have supported these early findings. They found that norfloxacin has weak affinity for linear or relaxed or gyrase-relaxed duplex DNA but does bind strongly to heat-denatured double stranded DNA (Shen and Pernet, 1985). They found this characteristic was independent of G + C content of the target DNA (Shen *et al.*, 1989b). They also showed that poly(dG) substrates were preferentially bound over the other nucleotides including inosine. It was reasoned since guanosine has the potential for two hydrogen bonding donors whereas the other bases only have one, norfloxacin interacts with single stranded DNA via hydrogen bonding. This is supported by the five-fold decrease in norfloxacin binding to poly(dI). Inosine is identical to guanosine except for it lacks the amino group on the purine ring which is a hydrogen bond donor in complementary strand pairing. Quinolones do interact with double stranded DNA, but

only with negatively supercoiled covalently closed circular templates (Shen and Pernet, 1985). This interaction was saturable, occurred at a given drug's supercoiling inhibitory concentration, and binding affinities were proportional to the drug's supercoiling inhibitory concentration. In addition, this binding was found to be cooperative (Shen *et al.*, 1989b). Furthermore, binding of DNA gyrase to relaxed DNA under conditions that prevented supercoiling enhanced quinolone binding (Shen *et al.*, 1989d). The kinetics of this interaction were similar to those observed with quinolone binding to a supercoiled substrate, ie., saturable and cooperative.

On the basis of these observations it was proposed that the binding site for quinolone drugs is formed in the gyrase-DNA complex during the ATP-requiring gate-opening step, which leaves 4 bp staggered cuts in the DNA (Shen et al., 1989c). In this model, the separated single stranded DNA segments form a 'bubble' similar to a denatured DNA structure to which the drug binds. High affinity binding arises from the self-association of two quinolone molecules yielding a 'supermolecule' with sufficient hydrogen bond acceptors to saturate the binding site. The implication of such a model is that the quinolone molecule has three functional domains: the DNA binding domain, a drug self-association domain, and gyrase interaction domain. Another similar model of quinolone interaction proposed by Yoshida et al. (1993) involves a quinolone pocket formed by gyrase-DNA interactions and that the drugs exert their actions during the DNA cleavage-renunion step. They also found that the affinity of enoxacin binding correlated well with the susceptibility of the gyrases for both the A and B subunits. A fourth model proposes that the C-7 and N-1 substituents on the quinolones interact with the enzyme, in contrast to the Shen model (Palumbo et al., 1993), the rings stack with DNA bases and the

carboxyl and carbonyl groups complex Mg^{2+} ions forming a bridge with the phosphate groups of the phosphodiester backbone.

1.21 Outer membrane changes associated with quinolone resistance

In addition to gyrase-mediated mechanisms, quinolone resistance may also result from altered drug permeation or via an efflux system, abeit at lower levels (reviewed in Hooper and Wolfson, 1993). Changes in the outer membrane affecting quinolone permeation have been described for *E. coli* (Cohen *et al.*, 1989; Hooper and Wolfson, 1993), *P. aeruginosa* (Iyobe *et al.*, 1991; Hirai *et al.*, 1987), *Enterobacter* sp., *K. pneumoniae*, *S. paratyphi*, *S. marcescens*, and *C. freundii* (cited in Hooper and Wolfson, 1993). In order for the quinolone antibacterials to be effective, they must cross membrane barriers to reach their targets in the cytoplasm. In the case of Gram negatives, this means traversing both the outer and cytoplasmic membranes. In Gram positive bacteria, they need only cross one membrane. For Gram negative organisms, the principal route of entry is via porins in the outer membrane (Chapman and Georgopapadakou, 1988). This is supported by the finding that OmpF-deficient *E. coli* were more resistant to quinolones than wild-type (Hirai *et al.*, 1986a; Hirai *et al.*, 1986b; Hooper *et al.*, 1986) although differences were no greater than four-fold and strains lacking OmpC were no different from wild-type in susceptibility. This is probably due to the differences in size between the two porins - 1.2 nm for OmpF versus 1.0 nm for OmpC - and the consequent ability of quinolones to pass through (Mortimer and Piddock, 1991); with quinolones being able to cross into the periplasmic space more freely via OmpF. In addition to alterations in OmpF expression, other mutations that have been associated with increased resistance to

quinolones were identified in *nfxB, norB, norC, nfxC, cfxB,* and *marA* (Hooper and Wolfson, 1993) although these all also exhibit decreased expression of OmpF.

The relative hydrophobicity of the quinolone in question may in fact determine which route the antibiotic uses to enter the cell and how it's MIC profile is affected in a porin-deficient host. Chapman and Georgopapadakou (1988) found that fleroxacin uptake in porin-deficient *E. coli* exhibited a nonporin pathway of entry. In the presence of Mg^{2+}, there was an increase in permeation which was analagous to an aminoglycoside or polymyxin B mechanism (Hancock, 1984). These compounds act by complexing the divalent cations assoicated with lipopolysaccharides which exposes hydrophobic regions on the outer membrane surface, allowing these drugs to diffuse through (Martin and Beveridge, 1986; Vaara and Vaara, 1983). This ability of quinolones to diffuse via a nonporin pathway increases with the hydrophobicity of the compound, which is the reverse of what would be expected with a porin-associated mechanism. Another finding was that rough mutants lacking the complex carbohydrate moieties on the cell surface exhibit lower MICs than wildtype (Hirai *et al.*, 1986a). These observations taken together may account for the increased intrinsic resistance to quinolones of the campylobacters in general. How the drug traverses the inner membrane is even less well understood and it may either be an energy-requiring process or a passive one.

The fact that *C. fetus* subsp. *fetus* are naturally resistant to nalidixic acid to high levels without cross-resistance to fluoroquinolones, makes it of interest to identify and characterize this mechanism. Cloning and sequencing of the *gyrA* gene from *C. fetus* would determine whether the resistance mechanism were related to alterations in gyrase leading to susceptibility to quinolone antibiotics. In addition, assays designed to determine protein function in the presence of nalidixic acid and the accumulation of this antibiotic in

cells would contribute to a more cohesive view of this resistance mechanism. Another aspect that will be examined is the degree of relatedness between *C. fetus* and *C. jejuni* as identified by amino acid identity and to see if the proteins possess homologous regions. Specifically, if they share a stretch in the C-terminal region of the protein only identified in *C. jejuni, H. pylori* and *P. aeruginosa* to date. This thesis is directed at investigating these possibilities.

2. Materials and Methods

2.1 Bacterial strains

Campylobacter fetus subsp. *fetus* UA60 (ATCC 27374) was obtained from the Sick Children's Hospital in Toronto, Ontario and *C. fetus* strain UA1221 (LCDC 93-15515) was from the Laboratory Centre for Disease Control in Ottawa, Ontario. *C. jejuni* UA580 (LCDC L10-4, NCTC 11168) was also obtained from the LCDC. *C. coli* strain UA585 (118114^R) was obtained from the Public Health Lab Service, University Hospital Wales, Cardiff, United Kingdom. *Helicobacter mustelae* strain UA862 was obtained from Galaxo Group Research Limited, Middlesex, United Kingdom. UA60R8 is a laboratory mutant derived from UA60 which was selected for resistance to ciprofloxacin. *Escherichia coli* strains DH5α, DH10B and JM107 were obtained from Gibco/BRL Life Technologies (Bethesda, Maryland).

2.2 Bacterial culture media and reagents

All *Campylobacter* cultures were grown on MH (Mueller-Hinton [Unipath Inc., Basingstoke, England]) or BHI (Brain Heart Infusion [Unipath Inc.]) media. MH agar or BHI agar were prepared by adding 1.5% (w/v) agar (Life Technologies) to powdered media prior to autoclaving at 121°C and 15 psi for 15 minutes. Liquid cultures of *C. fetus* were grown in BHI supplemented with 5% Fetal Bovine Serum (Hyclone Laboratories, Logan, Utah). DH5α and DH10B were grown in LB (Difco Laboratories, Inc., Detroit, Michigan) broth with agar added to 1.5% for solid media. MH agar was supplemented with 24 μg/ml

of nalidixic acid (Sigma Chemical Co., St. Louis, Missouri) or ciprofloxacin (Miles Inc., West Haven, Connecticut). LB agar and broth were supplemented with 200 μg/ml of carbenicillin (Pyopen, Ayerst Laboratories, Montreal, Quebec) and 50 μg/ml of ampicillin (Penbritin, Ayerst Laboratories) respectively for propagating cells with plasmids expressing ampicillin resistance. Kanamycin (Sigma Chemical Co.) was added to either LB agar or broth to a final concentration of 25 μg/ml. Chloramphenicol (Terochem Laboratories, Ltd., Edmonton, Alberta) was added to a final concentration of 20 μg/ml in MH agar. Only MH agar was used for MIC determinations.

2.3 Bacterial culture conditions

Campylobacter cultures were incubated at 37^{0}C in 7% CO_2 on MH or BHI agar in a Napco E Series Model 5100 CO_2 incubator (National Appliance Company, Tualatin, Oregon) or in a 3.5 litre Oxoid American polycarbonate anaerobic jar (Unipath Inc.). A Campylobacter Gas Generating Kit (Unipath Inc.) was placed in the jar following manufacturer's instructions or air in the jar was evacuated with a Speedivac High Vacuum Pump Model 2SC20A (Edwards High Vacuum Limited, Sussex, England) to -80 kPa and replaced with a CO_2 gas mixture (5% CO_2, 5% H_2 and 90% N_2 [Linde, Mississauga, Ontario, Canada]). Large-scale *C. fetus* broth cultures were inoculated with 1 ml of a cell suspension from fresh plates and grown as a stationary culture in a Corning 2.8 L culture flask (Corning Science Products, Toronto, Ontario) in a CO_2 incubator for 36-48 hours. Some broth cultures were also grown in 500 ml culture bottles (Life Technologies) with 200 ml of BHI inside anaerobic jars in a New Brunswick Scientific Controlled Environment Incubator Shaker Model G-25 (New Brunswick Scientific Co., Inc., Edison, New Jersey) at

37^{0}C and 100-140 rpm. Small-scale cultures were grown in 17 x 100 plastic culture tubes (Simport, Beloeil, Quebec) placed inside anaerobic jars in a shaking incubator overnight. Plate cultures were transferred to fresh media at least every 7 days. Cultures in anaerobic jars were infused with fresh gas mixture or a fresh Gas Generating Kit inserted every 48 hours. *E. coli* plate cultures were maintained at 37^{0}C in a dry air incubator. Large scale *E. coli* broth cultures were grown in 1 litre Erlenmeyer flasks in a shaking incubator at 150 rpm and small scale broth cultures were grown in plastic culture tubes in a New Brunswick Scientific Model TC-7 tube roller (New Brunswick Scientific Co., Inc.).

2.4 Cloning Vectors and sequencing primers

DNA cloning vectors used to clone the UA60 *gyrA* as well as related PCR products were pUC13, pUC19 (Life Technologies), pUC20 (Boehringer Mannheim, Laval, Quebec) or pBluescript II SK+/- (Stratagene Cloning Systems, La Jolla, California). Recombinant derivatives of these plasmids that were used for sequencing were primed with either forward and reverse pUC/M13 universal primers (Promega Corporation, Madison, Wisconsin; New England Biolabs, Inc., Beverly, Massachusetts; University Core DNA Services, University of Calgary, Calgary, Alberta) for pUC-based plasmids and pBluescript-based plasmids or T3/T7 sequencing primers (Stratagene Cloning Systems, La Jolla, California or DNA Synthesis Laboratory, University of Alberta, Edmonton, Alberta) for pBluescript-based plasmids. PCR primers were also utilized for sequencing and these were column-purified with BioRad Biospin 6 columns (BioRad Laboratories, Ltd., Richmond, California) and their concentrations were adjusted to 1.5 pmol/µl.

2.5 DNA Isolation

DNA cloning vectors and some gyrase plasmid clones were isolated using a modification of the large scale, alkaline denaturation procedure of Birnboim and Doly (1979). Briefly, the bacteria in overnight cultures were sedimented at 4, 000 xg in a JA-14 rotor. The bacterial pellet was resuspended in 2.5 ml of solution I (0.25 M Tris [pH 8.0], 50 mM glucose, 10 mM EDTA). 5 ml of solution II (0.2 N NaOH, 1% SDS) was added and the mixture was swirled until it became clear and incubated for a maximum of 5 minutes at room temperature. This step was usually terminated after 3 minutes. 3.75 ml of solution III (3 M CH_3COONa [pH 5.2]) was added and the tubes were gently agitated until a white precipitate formed. This material was centrifuged at 18, 500 xg for 15 minutes to sediment the cellular debris. The supernatant was precipitated with 2.5 volumes of 95% ethanol at -20^{0}C for 1 hour or overnight. The DNA was collected by centrifugation at 14, 000 xg, the supernatant was decanted, and the sedimented DNA air-dried. The pellet was redissolved in 10 ml of TE buffer and Rnase A was added to a final concentration of 5 µg/ml. 10 g of $CsCl_2$ was dissolved in the solution and this was transferred to a 13.5 ml Ultraclear Quickseal ultracentrifuge tube (Beckman). EtBr was added to saturating concentrations (usually 150 µl of a 10 mg/ml stock solution) and the tube heat sealed. Density gradient centrifugation was carried out at 65, 000 rpm for 4.5 hours in a NVT-65 near vertical rotor (Beckman) or at 55, 000 rpm for 20 hours in a 70.1Ti fixed angle rotor (Beckman) in a Beckman L-80 Ultracentrifuge. The plasmid DNA band was visualized with a Woods Lamp set to 366 nm and removed with a long tipped pasteur pipette passed through the neck of the tube. EtBr was removed by extracting the mixture 4 times with isoamyl alcohol. The aqueous phase was precipitated with 70% ethanol or diluted 1:3 and

precipitated with 95% ethanol. DNA concentrations were estimated on an agarose gel against a known standard.

For sequencing, ligations, endonuclease restriction, deletions and transformation, DNA was prepared by a modified mini-preparation procedure which was an adaptation of the large-scale alkaline denaturation method. The alkaline denaturation step was only performed for 3 minutes, which appeared to enhance plasmid DNA recovery, and volumes for each solution were scaled down to 100 µl, 200 µl and 150 µl respectively for each step. Subsequent to ethanol precipitation after step three, DNA was either washed with cold 70% ethanol; phenol:chloroform:isoamyl-alcohol (25:24:1 v/v) extracted; applied to a Chromaspin 1000 + TE column (Clontech Laboratories, Inc., Palo Alto, California); or centrifuged through a Millipore Ultrafree-ProBind 0.45 µm filter (Millipore, Mississauga, Ontario). Alternatively, some plasmid DNA was prepared by the Qiagen-tip 100 (Qiagen Inc., Chatsworth, California) purification procedure. Cells were grown in 50 ml L-broth in 250 ml Erlenmeyer flasks with shaking at 37^{0}C. Cells were centrifuged as described previously and resuspended in 4 ml buffer P1 (100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). This mixture was denatured by adding 4 ml buffer P2 (200 mM NaOH, 1% SDS) and gentle swirling for 5 minutes. Chromosomal DNA and other cellular debris was precipitated by adding 4 ml buffer P3 (3.0 M CH_3COOK, pH 5.5). The solution was clarified by centrifuging at 20,500 xg for 45 minutes in a JA-20 rotor. The supernatant was carefully applied to a Qiagen-tip 100 which had been equilibrated with 4 ml QBT buffer (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100). The solution was allowed to flow through the column by gravity. The column was washed by adding 2 x 10 ml QC buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0) and DNA was eluted with 5 ml QF buffer (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5).

DNA was precipitated with 0.7 volumes or 3.5 ml of isopropanol at room temperature. The mixture was centrifuged at 15, 000 xg for 30 minutes in a JA-20 rotor and the DNA pellet washed with 70% ethanol or the DNA reprecipitated with 95% ethanol without adding additional salt. DNA was usually redissolved in 500 µl of 0.1X TE yielding a DNA concentration of approximately 0.5 µg/ml.

UA60 and UA1221 chromosomal DNA was isolated using a modified procedure of Ezaki *et al* (1988). One inoculating loopful of cells from a fresh plate culture was resuspended in 1 ml of MH broth. After brief agitation, the cells were pelleted by centrifugation in a microcentrifuge at 16, 000 xg for 30 seconds. The supernatant was removed and replaced with 200 µl of isolation buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0), 10 µl 10mg/ml RNase A and 5 µl 20% w/v SDS. The tube was gently shaken until a homogeneous mixture was achieved and incubated at 65^0C for 5 minutes. 500 µl of a phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added to the hot mixture and the tube was gently inverted repeatedly to mix the sample. The microcentrifuge tube was centrifuged for 5 minutes at 16, 000 xg to separate the layers. The lower organic phase was removed with a pipet and discarded and the tube centrifuged again. The upper aqueous phase was extracted twice with an equal volume of chloroform and then precipitated with 2.5 volumes of ice-cold 95% ethanol and centrifuged to collect the DNA. The DNA pellet was washed with 500 µl of cold 70% ethanol, recentrifuged, and dried in a vacuum dessicator. The DNA was usually redissolved in 500 µl 0.1X TE buffer. This DNA was suitable for restriction endonuclease analysis, ligation and PCR amplification.

2.6 Agarose gel purification of DNA

Restriction endonuclease-digested plasmid or chromosomal DNA were obtained by electrophoresis in 0.8% - 1.0% Ultrapure agarose (Life Technologies) - concentration of agarose was dependent on the size of fragment to be purified with higher concentrations used for isolating smaller bands. Gels were run at 8 - 10 V/cm at room temperature with BioRad Mini-Sub Cell or Wide Mini-Sub Cell (BioRad) or Aquebogue Model 850 (Aquebogue Machine Shop, Aquebogue, New York) nucleic acid electrophoresis apparatus. Submarine agarose gels were run in 1X TAE buffer (40 mM Tris-HCl, 40 mM CH_3COOH, 1 mM EDTA). Gels were stained with a solution of 5 µg/ml EtBr in distilled H_20 for 10 - 15 minutes after electrophoresis and then visualized on a UV light source at a wavelength of 366 nm. Desired fragments were excised from agarose gels with a clean safety razor blade, as close to the boundaries of the fluorescing material as possible. DNA was purified from the agarose using the modified method of Koenen (1989). Gel slices were placed in a BioRad Prep-A-Gene spin filter (BioRad) and submerged in liquid nitrogen (Liquid Carbonic, Edmonton, Alberta) for 5 minutes. The filter unit was placed into a 1.5 ml microcentrifuge tube and centrifuged at 16, 000 xg for 5 min. DNA was either used directly for ligation or concentrated by ethanol precipitation. Some DNA samples were also purified by running in Ultrapure LMP agarose (Life Technologies). Gel slices were melted at 65^0C and the molten mixture extracted with phenol/chloroform (Sambrook *et al.*, 1989). DNA was precipitated with ethanol prior to use.

2.7 DNA ligation methods

Vectors pBluescript II SK+/-, pUC19 and pUCBM20 were used for cloning *gyrA* PCR products, subcloning the gyrase A gene and generating sequencing templates. Commercial sources of each cloning vector were transformed into DH10B and stock DNA solutions prepared from these. DNA was purified via $CsCl_2$ density gradient centrifugation to ensure they were free from chromosomal contamination. This DNA was adjusted to a concentration of about 0.2 µg/µl. This allowed standard amounts of plasmid to be used for each manipulation requiring cloning vectors. PCR products were treated prior to ligation by T4 polymerase blunting in the presence of all 4 dNTPs (12.5 µM final concentration of each dNTP) at room temperature. Vectors were linearized with a restriction enzyme that left blunt ends, such as *EcoR*V, *Hind*II and *Sma*I. Linearized vector DNA was treated with Shrimp Alkaline Phosphatase (USB) following the manufacturer's recommendations. Concentrations of both insert and vector DNA were adjusted so they were about equal on the basis of using 4 µl of cut vector DNA. Ligation volumes were 20 µl with commercial ligase buffer and 1 U of T4 DNA ligase. Ligations were carried out at room temperature for 1 to 3 hours and 5 µl of the ligation mixture was used to transform DH10B. DNA fragments with staggered ends were ligated in reactions using 3:1 insert-to-vector ratios and 0.2 µg of vector DNA. If possible, DNA was cut with enzymes that left incompatible ends. If this was not possible, then vector DNA was treated with Shrimp Alkaline Phosphatase to reduce background.

2.8 DNA Transformation

DNA was transformed using a modified $CaCl_2$ procedure of Cohen *et al.* (1972) and Seidman (1987). Cells were grown overnight in 5 ml broth culture in a test tube with

rolling at 37^{0}C. 200 µl of the overnight culture was used to inoculate 400 ml of LB in a 1 litre Erlenmeyer flask and grown with shaking for three or four hours at 37^{0}C. Cells were briefly cooled on ice and then sedimented in a Beckman JA-14 rotor in a J2-21 High Speed Centrifuge (Beckman Instruments, Palo Alto, California) at 3, 110 xg for 10 minutes. All subsequent steps, including centrifugation were carried out on ice or at 4^{0}C. Culture supernatants were poured off after centrifugation and the cell pellet was gently resuspended with 10 ml of $CaCl_2$ solution (60 mM $CaCl_2$, 10 mM 1,4-piperazinebis-(ethanesulfonic acid) (PIPES) [pH 7.0], 15% glycerol). The cells were incubated for 30 minutes on ice, then centrifuged for 30 seconds at 3, 110 xg. The cell pellet was resuspended with fresh $CaCl_2$ solution to a turbidity equivalent of a McFarlane standard #8 with fresh $CaCl_2$ solution (equivalent to 10^{10} CFU/ml). The cell suspensions were divided into 200 µl portions in sterile 1.5 ml microcentrifuge tubes and frozen in liquid nitrogen and stored at -80^{0}C until needed. Prior to transformation, cells were thawed on wet ice, DNA to be transformed was added and the mixture was gently mixed by agitating the tube and then incubated for 20 minutes on ice. This was followed by a 45 second heat pulse at 42^{0}C. The transformation mixture was quickly cooled for 5 minutes on ice prior to plating the bacteria on appropriate selective medium. Plasmids with either kanamycin or chloramphenicol resistance determinants were pre-grown at 37^{0}C with an additional 200 µl of MH broth added. Typical transformation volumes were 100 µl of cells and 5 µl of ligation mixtures.

2.9 DNA hybridization

To identify and characterize plasmid clones or gyrase fragments from chromosomal digests, DNA hybridization by the method of Southern (1975) was utilized. DNA to be screened was digested and electrophoresis was performed on an agarose gel. The gel was stained with ethidium bromide and the resulting banding patterns were recorded using Polaroid Type 667 film (Polaroid, Rexdale, Ontario) or with a The Imager video-capture system using a CCD camera from Appligene (Appligene,) and thermal printouts (Mitsubishi, Somerset, New Jersey). The gel was immersed in denaturation solution (0.5 N NaOH, 1.5 M NaCl) and incubated for 30 minutes with gentle agitation. The denaturing solution was decanted and neutralization solution was added (1 M Tris-HCl [pH 5.5], 2 M NaCl) and incubated a further 30 minutes with shaking. The gel was equilibrated for 5 minutes in 20X SSC and then placed on top of a sheet of Whatman 3MM Chr paper soaked in 20X SSC on a glass plate with each end of the paper overhanging into a baking dish with 20X SSC. A piece of Hybond-N nylon membrane (Amersham Life Science Inc., Arlington Heights, Illinois) or MSI Nitroplus nitrocellulose membrane (Micro-Separations, Inc., Westboro, Massachussets) was cut to the same dimensions as the gel to be blotted with a 5 mm overlap on either edge and placed on top of the gel. 4 pieces of 3MM Chr paper were placed on top of these and then a 1/2 inch layer of paper towels. A glass plate to distribute weight evenly was placed on the paper towels and about 1 kg of weight was placed on top. The transfer was carried out for at least 12 hours. The membrane was marked with the positions of the wells and the edges of the gel and rinsed for 5 minutes in 2X SSC and allowed to air dry. DNA was fixed to the nylon by UV irradiating the saran wrapped membrane in a Stratagene Cross-linker (Stratagene) using the auto cross-link feature.

For chemilumiscent detection, membranes were incubated at 37^{0}C in hybridization buffer (50% [v/v] formamide, 5X SSC, 2% [w/v] blocking reagent, 0.1% [w/v] N-lauroylsarcosine, 0.02% [w/v] SDS) without agitation in a plastic tupperware dish. For radioactive detection, membranes were sealed in plastic hybridization bags with 10 ml hybridization buffer (50% [v/v] formamide, 5X SSC, 0.1% [w/v] SDS, 1 mM EDTA, 0.02% [w/v] ficoll, 0.2% [w/v] BSA, 0.2% [w/v] polyvinyl pyrrolidone). DNA probes were synthesized via both random primer (Feinberg and Vogelstein, 1983) and nick translation (Sambrook *et al.*, 1989) methodology by incorporating DIG-labelled dUTP or [α^{32}P]dCTP. In random primer labelling, labelling mixes were comprised of 1 mM each of dATP, dCTP, and dGTP and 0.65 mM dTTP and 0.35 mM DIG-labelled dUTP or 1 mM each of dATP, dTTP, and dGTP and 2 µM [α^{32}P]dCTP. Nick translation mixes were 100 µM each of dATP, dCTP, and dGTP and 65 µM of dTTP and 35 µM of DIG-dUTP or 100 µM each of dATP, dTTP, and dGTP and 2 µM of [α^{32}P]dCTP. The hybridization mixture consisted of probe and hybridization mixture was heated at 95^{0}C for 10 minutes and then added to the blot to be hybridized. Hybridization was carried out at 37^{0}C for one or two hours. The hybrization solution was saved for later use and the blot washed 2 x 5 minutes with 2X SSC, 0.1% (w/v) SDS at room termperature. They were then washed 2 x 15 minutes in 0.1X SSC, 0.1% (w/v) SDS at 65^{0}C. For chemiluminescent detection, the blots were then briefly washed in DIG wash buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% [v/v] Tween 20) for 5 minutes. The blot was then incubated in DIG buffer 2 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 1% blocking reagent [Boehringer Mannheim]) at room temperature with gentle agitation for 30 minutes. The liquid was poured off and 20 ml of fresh buffer 2 containing 75 mU/ml anti-DIG-Alkaline Phospatase conjugate was added. The membrane was incubated a further 30 minutes at room temperature with gentle agitation. The

antibody-treated blot was washed with 100 ml wash buffer at room temperature and then equilibrated with buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 minutes. 10 ml of a 0.1 mg/ml Lumigen PPD (Boehringer Mannheim) in buffer 3 solution was added and the membrane was incubated for 5 minutes. Visualization of blots was achieved by chemiluminescent exposure of Kodak XAR-5 X-ray film (Kodak, Toronto, Ontario). For radioactive detection, blots were checked for counts using a hand-held Geiger-Mueller counter and then wrapped in saran wrap prior to exposure to X-ray film in a cassette holder. Exposure times varied with the intensity of radiation detected with the G-M counter.

A modified in-gel hybridization technique of Ehtesham and Hasnain (1991) was also utilized when screening for inserts or when a quick result was desired. Briefly, agarose gels were prepared as normal, EtBr stained, and the results were recorded with Polaroid film or video-captured. Without any pre-treatment gels were dried onto Whatman 3MM Chr paper in a gel dryer for 30 minutes under low heat. The dried gels could be stored indefinitely at room temperature until used. Prior to hybridization the gel was immersed in distilled H_20 to remove the paper and then transferred to either a plastic dish or hybridization bag and incubated with heat-denatured radioactive probe solution. There was no need for pre-hybridization since the agarose did not contain any substances that would interfere with the probe. After hybridization, usually from 1 h to overnight, the gel was washed using the regular membrane hybridization protocol previously described for radioactive hybridization, excess liquid was removed, and the hybridized gel was wrapped in saran wrap and exposed to X-ray film in a cassette holder.

2.10 Colony screening

Two detection methods were also utilized for colony hybridization. Either [^{32}P] or DIG- labelled DNA were used as probes. If radioactivity was used, Nitroplus nitrocellulose membrane discs (MSI) were used. For DIG-labelled probes, Hybond-N nylon membrane discs (Amersham) were used. In both methods, colonies were replicated onto identical plates, one of which had a sectored membrane disc on the agar surface. Colonies were picked using toothpicks from the experimental plate and touched to each plate. After overnight growth, the petri dish without the membrane was kept as a template and the membrane was lysed, denatured and hybridized. Membranes were first incubated for five minutes in just enough 10% SDS to wet the disc. After removing excess fluid from the membrane, the disc was transferred to a petri dish containing denaturing solution (1.5 M NaCl, 0.5 N NaOH) and incubated 10 minutes. Excess liquid was removed and the disc was transferred to another dish containing neutralizing solution (3 M NaCl, 1 M Tris-HCl, pH 5.5) for a further 10 minutes. A final incubation for 10 minutes in 2X SSC was performed and then the membrane was air-dried on 3MM paper. The DNA was either baked to the membrane at 65^{0}C for 1 hour or UV fixed. Discs were probed, washed and exposed in the same fashion as Southern hybridizations were performed.

2.11 Deletion mutant generation

For sequencing, deletion mutants were generated from plasmids to create overlapping fragments. One method involved digestion of the desired fragment with restriction enyzmes and ligation of the resultant fragments into pBluescript. This was used in addition to exonuclease III digestion (Henikoff, 1984). For exonuclease digestion,

plasmids were doubly cut with an enyzme that left a 3′ protruding end and an enzyme that left a 3′ recessed end. The 3′ protruding termini are resistant to exonuclease III digestion, thus protecting DNA duplexes from degradation and 3′ recessed termini are susceptible to exonuclease digestion. In this way, it was possible to create directional mutants and orient recombinant plasmids for sequencing since at least one end of the insert is always constant. If the plasmids allowed it, *Pst*I (Life Technologies) was the enzyme of choice for generating the 3′ protruding ends and restriction endonuclease *Not*I (Pharmacia) was favored for leaving exonuclease sensitive ends. In other constructs, due to the absence of these sites or a desire to generate deletions in the opposite direction, *Sac*I (Boehringer Mannheim) or *Kpn*I (Life Technologies) were used instead of *Pst*I. In these circumstances, *Sma*I or *Cla*I (Boehringer Mannheim) were utilized to leave blunt ends or 5′ overhangs, respectively, as exonuclease substrates. 2.5 µg of DNA and 150 U of exonuclease III (Life Technologies) was used for every six timepoints that were to be taken. In a procedure where 12 aliquots were to be taken, plasmid DNA was diluted in 1X exonuclease III buffer (66 mM Tris-HCl [pH 8.0], 0.66 mM $MgCl_2$) in total volume of 30 µl. These deletion reactions were carried out at 30^0C and aliquots were removed every 30 seconds. Six successive fractions were pooled together yielding two groups and each group was added to a microcentrifuge tube containing 45 µl of S1 nuclease buffer (40 mM CH_3COOK [pH 4.6], 0.33 M NaCl, 1.35 mM $ZnSO_4$) and 12 U of S1 nuclease (Life Technologies). The S1 reaction proceeded for 30 minutes at room temperature and was terminated by adding 6 µl of S1 stop buffer (0.3 M Tris base, 0.05M EDTA) and heating to 70^0C for 10 minutes. 6 U of Klenow (Life Technologies) in 6 µl of Klenow buffer (20 mM Tris-HCl [pH 8.0] 10 mM $MgCl_2$) was added and the tubes were incubated for 3 minutes at 37^0C. 6 µl of dNTP mix (0.125M each of dATP, dTTP, dCTP, and dGTP) was added and the tubes were incubated a

further 5 minutes. DNA was concentrated by ethanol precipitation as previously described and collected by centrifugation. Nucleic acid pellets were air-dried and redissolved in 20 µl of ligation mixture and incubated at room temperature for at least 1 hour. 5 µl was used for transforming DH10B in the previously described procedure and the cells were plated on carbenicillin-containing LB agar.

2.12 DNA Sequencing

DNA sequencing of *gyrA* was carried out using the dideoxy chain termination method (Sanger *et al.*, 1977) with either Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) by a modified method of Wang (1990) or *Taq* DNA polymerase cycle sequencing (Life Technologies). Template DNA was always double-stranded plasmid or double-stranded PCR products. Numerous plasmid DNA isolation methods were assessed for generating template. DNA prepared by $CsCl_2$ density gradient centrifugation was used directly without any further treatment or purification. Some crude plasmid preparations isolated by a mini-Birnboim and Doly method were extracted with organic solvents, RNase A-treated (Pharmacia, Baie d'Urfé, Quebec) and then ethanol-precipitated prior to sequencing. In other instances, crude plasmid DNA pellets were washed with 70% ethanol and RNase-treated or only RNase-treated prior to cycle sequencing. 5 µg of double-stranded plasmid DNA was routinely used in Sequenase sequencing protocols, with adjustments made for the size of the plasmid and 1 µg or less for cycle sequencing. This amount corresponds to approximately two picomoles of template DNA of 4 kb in size.

For Sequenase protocols, reactions were radioactively labelled by [α^{35}S]dATP (New England Nuclear, Boston, Massachusetts) incorporation in a two step procedure. Briefly, template DNA was denatured by 0.02 N NaOH treatment for 5 minutes followed by ethanol precipitation .or heating to 90^{0}C for 10 minutes immediately followed by cooling in an 95% ethanol/dry ice bath. Alkaline denaturation resulted in stronger signals but heat denaturation was used when a quick method was desired. 4 to 6 pmol of sequencing primer were annealed to the denatured template in a volume of 10 µl at 37^{0}C in Sequenase buffer (40 mM Tris-HCl, 20 mM $MgCl_2$, 50 mM NaCl, pH 7.5). The annealing reaction was allowed to continue for at least 15 minutes or until the other mixtures were prepared. To the annealing mixture, 1 µl of 0.1 M DTT; 2 µl of a 1:5 dilution labelling mix (1.5 µM each of dGTP, dCTP and dTTP); 1 µl [α^{35}S]dATP; and 2 µl of a 1:8 dilution of Sequenase were added. The labelling reaction was carried out at room temperature for 5-10 minutes, longer if sequence further from the primer was desired, 3.5 µl of this mixture was aliquoted into each of 4 tubes containing 2.5 µl of one of the termination mixtures (80 µM of each dNTP and 50 mM NaCl, as well as 8 µM of ddATP, ddCTP, ddTTP or ddGTP in the A, C, T or G mixes respectively). These were incubated at 37^{0}C for 5 minutes and terminated by adding 4 µl of stop solution (95% [v/v] formamide, 0.1% [w/v] bromophenol blue, 0.1% [w/v] xylene cyanol). Reactions were stored at -20^{0}C until used.

Cycle sequencing was carried out with *Taq* polymerase in a Perkin-Elmer Model 480 thermocycler (Perkin-Elmer Cetus Corp., Emeryville, California). Template DNA amounts were of 0.5 µg or less with the quality of DNA used ranging from $CsCl_2$-purified to crude mini-preps. Sequencing primers were 5′ end-labelled using T4 polynucleotide kinase (Life Technologies) and [γ^{33}P]ATP (NEN) as recommended by the manufacturer with

modification. 1.0 to 1.5 pmol of primer, 0.5 µl of radionuclide (1 pmol), 1.0 µl 5X Kinase buffer (300 mM Tris-HCl [pH 7.8], 50 mM $MgCl_2$,, 1.65 µM ATP, 75 mM 2-mercaptoethanol), Milli-Q H_20 and 1.0 U of T4 kinase. The labelling reaction was incubated for 10 minutes at 37^0C and was then transferred to 55^0C for an additional 5 minutes. To this 5 µl volume, template DNA, 4.5 µl of 10X *Taq* cycle sequencing buffer (300 mM Tris-HCl, 50 mM $MgCl_2$, 300 mM KCl, pH 9.0), 0.5 µl *Taq* polymerase and MilliQ H_20 obtained from a Millipore model Deionizer (MilliQ) to a final volume of 36 µl was added. The solution was mixed and 8 µl was added to each of four 0.6 ml microtubes with one of the termination mixtures (100 µM of each dNTP and either 2 mM ddATP, 1 mM ddCTP, 2 mM ddTTP or 0.2 mM ddGTP for A, C, T or G mixes respectively). For universal sequencing primers, the standard sequencing parameters were 94^0C denaturation for 30 seconds, 50^0C annealing for 30 seconds, and 72^0C extension for 45 seconds through 40 cycles. Other primers had annealing temperatures that reflected the size and specificity of these oligonucleotides. Reactions were terminated by adding 4 µl of stop solution. *Taq* DNA polymerase was also used in a two-step sequencing protocol with [α^{35}S]dATP incorporation when template regions were GC rich. Conditions were identical to those used with Sequenase except the extension step was carried out at 70^0C in a Temp-Blok and buffers were optimized for *Taq*.

Electrophoresis of sequencing samples was carried out with a BioRad Sequi-Gen II Sequencing Cell (BioRad) in either the 21 x 50 cm or 38 x 50 cm formats or a BRL Model S1 Sequencing Cell System (Life Technologies) sequencing apparatus. Sequencing gels were composed of 5% or 6% (w/v) 19:1 or 29:1 acrylamide/bis-acrylamide (BioRad) mixtures, 8M urea and 1X TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and electrophoresis was performed in 1X TBE buffer. Gels were polymerized with the addition

of 0.1% ammonium persulfate and 3 mM TEMED (final concentrations). Electrophoresis was carried out at constant power to maintain a temperature of 50^0C which was monitored with termperature indicator stickers on the outside of the glass plates. After electrophoresis was completed, the silanized plate was removed and the gel lifted from the non-treated plate using Whatman 3MM Chr paper cut to size. Saran Wrap was placed over the gel and dried, gel side up, in a gel dryer. Gels were dried at 80^0C for 1 hour. Dried gels were placed into an X-Ray cassette holder with a sheet of Kodak Bio-Max X-ray film (Kodak) with the gel side against the surface with the emulsion and exposed overnight or longer depending on the level of radiation detected with a handheld Geiger-Mueller counter. Autoradiograms were developed in an automated Kodak developer.

2.13 PCR amplification

All thermocycling reactions were carried out in a Perkin-Elmer Model 480 thermocycler (Perkin-Elmer Cetus) using thin-walled 0.6 ml microcentrifuge tubes. All denaturation steps were performed at 94^0C for 30 seconds and all extensions were performed at 72^0C. Annealing temperatures varied with the primers used but length of this step was always 30 seconds. These termperatures were estimated using the general rule T_m - 5^0C, where T_m is approximated with the formula, $2^0C \times (N_{A/T}) + 4^0C \times (N_{G/C})$, where ATGC correspond to the four different bases. If inosine was included for degenerate oligonucleotides, a 1^0C decrease in T_m per inosine residue was assumed. Volumes were either 50 or 100 µl and reaction mixtures were overlayed with 20 or 50 µl of mineral oil respectively. *Taq* DNA polymerase was either purchased from BRL or from Promega and 2.5 U was used per reaction. dNTP stocks were made up to a final concentration of 2 mM

of each deoxynucleoside triphosphate from 100 mM stocks (Boerhinger Mannheim Canada or Pharmacia). PCR buffers supplied by the manufacturers were used as directed.

2.14 PCR Colony Screening

PCR was used for quick screening of transformants resulting from cloning or deletion mutant generation. Single colonies from overnight plates were picked with sterile toothpicks. They were then transferred to a labelled master plate containing the same antibiotic used in the original selection procedure and then to a 0.6 ml microcentrifuge tube containing PCR reagents (2 pmol of both forward and reverse primers [T3/T7 or pUC/M13], 1X PCR reaction buffer [20 mM Tris-HCl, 50 mM KCl, pH 8.4], 1.5 mM $MgCl_2$, 200 µM of each dNTP, Milli-Q H_20 and 2.5 U *Taq* DNA polymerase [Life Technologies]) in a 50 µl volume. The reaction was overlayed with 30 µl of mineral oil. The tubes were placed in the thermocycler and PCR carried out with a reaction profile of: 95^0C for 5 minutes for 1 cycle; 94^0C for 30 seconds, 50^0C for 30 seconds, 72^0C for 45 seconds for 35 cycles; and 72^0C for 5 minutes.

2.15 Gyrase protein isolation

The gyrase holoenzyme was isolated from UA60 by modified methods described by Gootz and Martin (1991) and Staudenbauer and Orr (1981). One litre cultures were grown for 48 h in culture flasks. Cells were collected by centrifugation in a JA-14 rotor at 3, 110 xg for 10 minutes. The cell pellet was resuspended in 1 ml of ice-cold TGED buffer (20 mM Tris-HCl [pH 7.5], 0.1 M KCl, 0.2 mM EDTA, 10% [w/v] glycerol, 5 mM dithiothreitol, 10 mM sodium metabisulfite, 1 mM phenyl-methylsulfonyl fluoride [PMSF])

per gram of cells. The suspension was collected in a 30 ml Corex centrifuge tube and placed on ice. The cells were sonicated with 15 x 30 second pulses with a Fisher Sonic Dismembrator Model 3000 (Fisher Scientific, Nepean, Ontario) on ice. Each sonicating pulse was interspersed with an equal number and period of incubations on ice. After sonication, the material was centrifuged for 30 minutes at 12, 100 xg in a JA-20 rotor. The cleared sonicated material was mixed with an equal volume of TGED-washed Heparin-agarose (BioRad), gently mixed, and incubated overnight at 4^0C. The mixture was poured into a 2.5 cm i.d. x 10 cm Bio-Rad Econo-column and washed with 4 x 20 ml TGED. Prior to enzyme elution, the column was washed with 3 column volumes of TGED containing 0.2 M KCl. The gyrase holoenzyme was eluted with 0.5 M KCl-containing TGED. 1.0 ml fractions were collected with a BioRad Model 2110 Fraction Collector (BioRad) in the drop collection mode and pooled prior to concentrating. For concentration in Centricon-30 microconcentrators (Amicon, Oakville, Ontario) the samples were first centrifuged in a JA-20 rotor at 3, 020 xg. Concentrated sample was dialyzed overnight against HEPES buffer (0.25 M HEPES [pH 8.0], 50 mM KCl, 1 mM dithiothreitol, 1mM EDTA, 10% [w/w] ethylene glycol) at 4^0C using 6, 000 to 8, 000 molecular weight cut-off (MWCO) Sprectra/Por diaylsis tubing (Spectrum Laboratory Products, Houston, Texas). The dialysate was applied to a novobiocin-Sepharose affinity column. Alternatively, cleared sonicated material was dialyzed immediately against HEPES buffer overnight and then directly applied to a novobiocin column. The novobiocin-Sepharose column was prepared from epoxy-activated Sepharose 6B (Pharmacia) as described by Staudenbauer and Orr (1981). The matrix was rehydrated in 100 ml distilled H_20 and washed on a sintered glass filter with 100 ml 0.3 M Na_2CO_3 (pH 9.5) The Sepharose was mixed with 15 ml 0.3 M Na_2CO_3 and 50 mg/ml novobiocin (Sigma) and incubated with gentle shaking at 37^0C overnight.

Excess groups were blocked with 1 M ethanolamine at 37^{0}C for 4 h. The derivatized matrix was successively washed with 300 ml each of 0.5 M NaCl, 0.3 M Na_2CO_3; distilled H_20; 0.5 M NaCl in 0.1 M CH_3COONa (pH 4.0); and distilled H_20. Prior to use, the matrix was poured into a 1.0 cm i.d. x 10 cm Econo-column (BioRad) and washed extensively with HEPES buffer. Gyrase was eluted with 2 column volumes of HEPES buffer containing 5M urea. Eluted material was dialyzed against TGED buffer at 4^{0}C overnight and then concentrated using Centricon-30 concentrators.

2.16 Supercoiling assay

Active gyrase enzyme was checked with a supercoiling assay described by Staudenbauer and Orr (1981). Plasmid pBR322 was relaxed with Topoisomerase I (BRL) in a 100 µl reaction volume for 3 hours at 37^{0}C. ATP was added to a final concentration of 10 mM and the DNA closed with T4 DNA ligase at room temperature for 1 h. Supercoiling assays were carried out at 30^{0}C in 35 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1.8 mM spermidine-HCl, 24 mM KCl, 0.14 mM EDTA, 5 mM DTT, 6.5% (w/v) glycerol, 1.4 mM ATP, 9 µg *E. coli* tRNA/ml, 0.36 µg BSA/ml.

2.17 Quinolone accumulation

Nalidixic acid accumulation was determined by the methods described by Chapman and Georgopapadakou (1988) and Mortimer and Piddock (1991). Cultures to be assayed were grown on solid media and then transferred to 5 ml of liquid media in 17 x 100 mm plastic culture tubes (Simport, Beloeil, Quebec), placed in an anaerobic jar and grown at 37^{0}C overnight in a shaking incubator. 1 ml of this overnight culture was

transferred to 750 ml of BHI broth + 5% serum in a 2.8 L culture flask or 750 ml of MH broth. The cultures were grown at 37^{0}C overnight in a CO_2 incubator. Cells were collected by centrifugation at 3, 110 xg with a JA-14 rotor and then washed with ice-cold 1X PBS and recentrifuged. The washed cells were resuspended in fresh PBS to a turbidity equivalent of a McFarlane Standard #8. 10 ml of these cells was equilibrated to 37^{0}C for 10 minutes in a 50 ml Nalgene Oakridge tube. Either 100 µl of a 10 mM stock solution of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for a final concentration of 100 µM CCCP or 95% ethanol was added in the CCCP- experiment. Nalidixic acid was added to a final concentration of 10 µg/ml. The cells were left at 37^{0}C and 500 µl aliquots were removed at 10 second intervals for 1 minute, then at 30 second intervals for another 4 minutes, and finally at 1 minute intervals for 5 more minutes. Each aliquot was dispensed into 1 ml of ice-cold PBS in a 1.5 ml microfuge tube, mixed and left on ice. After centrifugation, cells were washed once with PBS and then suspended in 1 ml of 0.3 M glycine-HCl and incubated overnight at room temperature with agitation. At this point, the tubes were covered in aluminum foil to prevent photo-bleaching. The next day, the cells were sedimented in a microcentrifuge and the supernatant was collected in 12 x 75 mm glass culture tubes for fluorescence determination. Tubes were kept covered until read in a spectrofluorometer. The excitation and emission spectra for nalidixic acid were 325 nm and 365 nm respectively.

2.18 Determination of MICs

The miniminal inhibitory concentrations of nalidixic acid and ciprofloxacin for UA60, , UA60R8, UA1221, UA580 and UA417 were determined using a modified method of Steers *et al.* (1955). Strains were originally grown on BHI agar overnight at 37^{0}C in 7%

CO_2, then incoculated into 3 ml of BHI + 5% fetal bovine serum (*C. fetus*) or MH broth (*C. jejuni* and *C. coli*). These were grown overnight and then sedimented at 3, 110 xg and resuspended in 1x PBS (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to an OD equivalent of a MacFarlane standard #6 (Difco Laboratories). 10 µl of this suspension was spotted onto MH agar containing exponentially increasing levels of quinolone antibiotics. The inital concentration for ciprofloxacin was 0.125 µg/ml which was doubled to the final concentration of 64 µg/ml which was tested. The initial concentration of nalidixic acid was 1 µg/ml which was doubled to the final concentration tested of 512 µg/ml. Plates were left at room temperature right-side-up to allow the spots to dry and then inverted and incubated at 37^0C in 5% CO_2. Growth was assessed after 24-36 hours.

3. Results

3.1 Cloning of UA60 DNA gyrase

UA60 chromosomal DNA was screened for *gyrA* by amplification of a 449 bp fragment (Figure 3.1) using primers GyrA1 (5′-GTIAGIGATGGITTIAAGCCIGTICA T-3′) and GyrA2 (5′-GTGIGGIGGTATGTTIGTIGCCAT-3′). These oligonucleotides were previously designed to identify the *C. jejuni* UA580 *gyrA* gene (Wang *et al.*, 1992). They are homologous to gene sequences coding for conserved amino acid sequences within the N-terminal region of gyrase proteins of several bacterial genera. The nucleotide sequence was deduced from the amino acid sequence on the basis of codon usage. Oligonucleotides GyrA1 and GyrA2 are designed from the peptides NH_2-VRDGLKPVH-COOH and NH_2-MATNIPPH-COOH respectively and are equivalent to amino acid sequences NH_2-ARDGLKPVH-COOH and NH_2-MATNIPPH-COOH in UA60. The region amplified from *C. fetus* spans nucleotides 428 to 877. Both *C. fetus* and *C. jejuni* PCR products were identical in size on a 1.0% TAE agarose gel (Figure 3.1). The fragment was blunted and ligated into pUC13. The insert was sequenced and this data used as the query sequence in a Blast search of the GenBank and EMBL nucleotide databases. From the search results it was determined the PCR product was amplified from *gyrA* on the basis of nucleotide sequence homology with the *gyrA* sequences from *C. jejuni* and other bacteria. The PCR product was ^{32}P-labelled and utilized as a probe and hybridized to a Southern blot of *C. fetus* UA60 DNA digested with *Bgl*II, *Dra*I, *EcoR*V, *Hind*III, *Hpa*I, *Nla*III, *Sac*I, and *Sph*I (Figure 3.2). A 5.5 kb *Sac*I and 5.9 kb *Hpa*I fragment was identified as was a single 2.7 kb *Bgl*II fragment. An attempt was made to subclone these fragments

Figure 3.1 Amplification of *gyrA* PCR products from chromosomal DNA.

PCR products amplified from *E. coli* JM107 (lane 2), *C. coli* UA585 (lane 3), *C. fetus* subsp. *fetus* (lane 4), *C. jejuni* UA580 (lane 5) and *H. mustalae* UA862 (lane 6). 1 kb marker is in lane 1 and lane 7 is the negative control. The arrow indicates the expected product of size ~449 in *C. fetus*. 5 µl of a 100 µl volume was loaded with bromophenol blue-xylene cyanol sample loading buffer on a 1.0% TAE agarose gel.

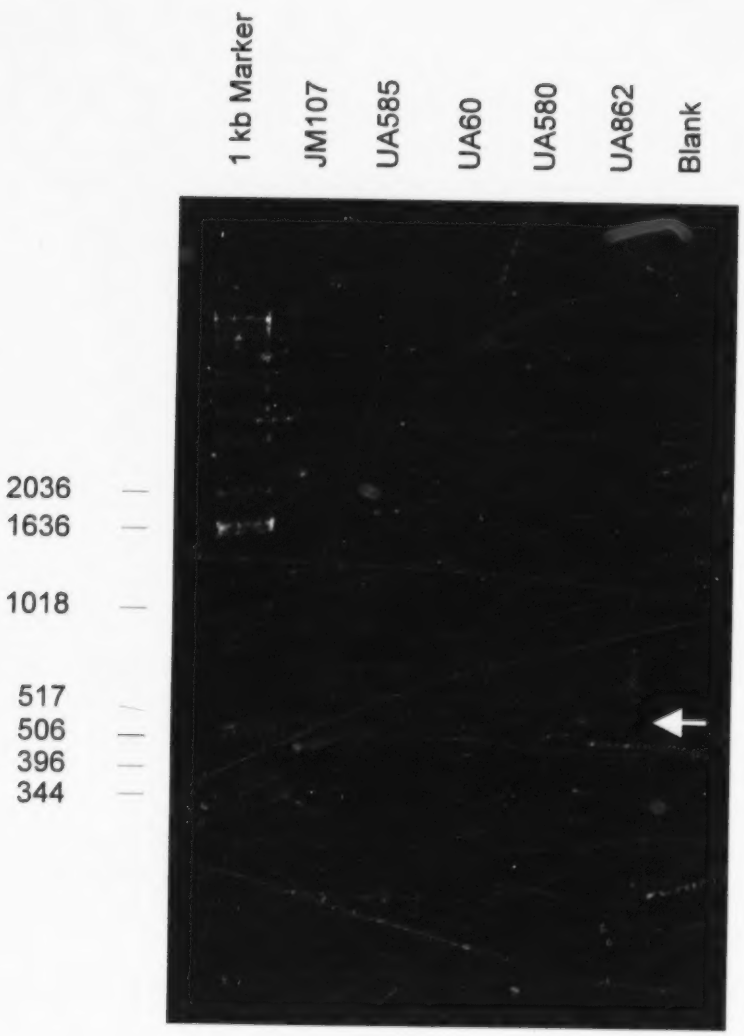
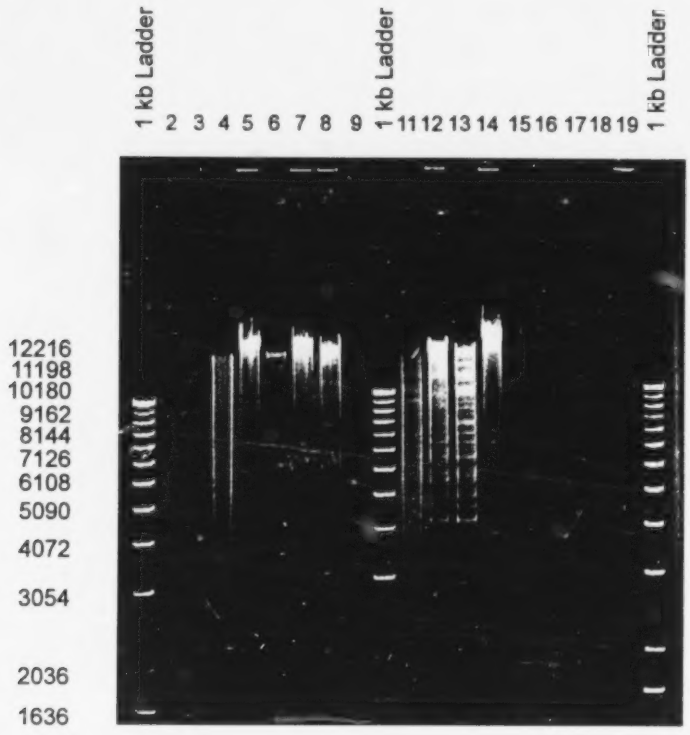

1 kb Marker
JM107
UA585
UA60
UA580
UA862
Blank
2036
1636
1018
517
506
396
344

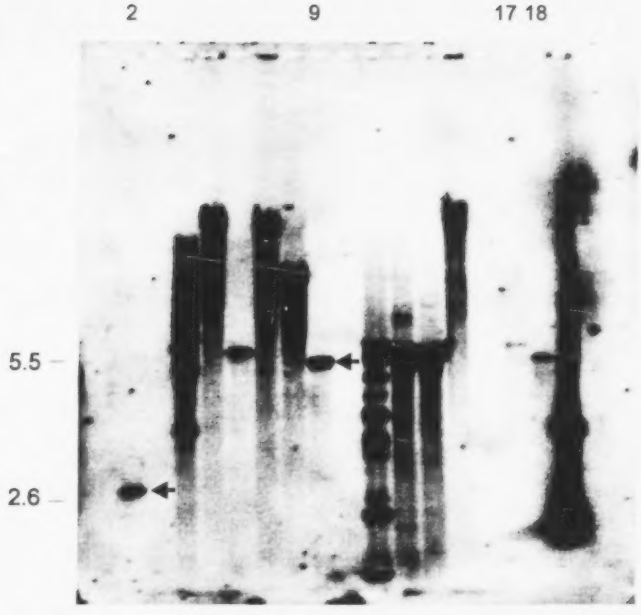
Figure 3.2. Hybridization of *gyrA* PCR probe to restricted UA60 chromosomal DNA.

(A) C. fetus UA60 chromosomal DNA digested with *Bgl*II (lane 2), *Dra*I (lane 3), *EcoR*V (lane 4), *Hind*III (lane 5), *Hpa*I (lane 6), *Nla*III (lane 7), *Sph*I (lane 8), *Sac*I (lane 9), and *Xba*I (lane 14) was separated by gel electrophoresis in 0.8% TAE agarose. DNA was also double digested with *Hind*III/*Hpa*I (lane 11), *Hind*III/ *Sac*I (lane 12) and *Hpa*I/ *Sac*I (lane 13) and purified fragments corresponding to those from *Bgl*II (lane 15), *Hpa*I (lane 16), and *Sac*I (lane 17) digests that hybridized with the UA60 *gyrA* PCR probe were also included. Lanes 1, 10, and 20 are 1 kb ladders; lane 18 is the UA60 *gyrA* PCR product cloned into pUC20 and lane 19 is pUC20. The Southern blotted gel was hybridized with the UA60 *gyrA* PCR probe (B) using DIG and chemiluminescent detection. The arrows indicate the 2.6 kb *Bgl*II fragment (lane 2) and 5.5 kb *Sac*I fragment (lane 9). The gel-purified fragments from chromosomal digests of *Hpa*I and *Sac*I also give the same banding pattern as the chromosomal digests (lanes 17 and 18, respectively).

A.


1 kb Ladder
2 3 4 5 6 7 8 9
1 kb Ladder
11 12 13 14 15 16 17 18 19
1 kb Ladder
12216
11198
10180
9162
8144
7126
6108
5090
4072
3054
2036
1636

B.

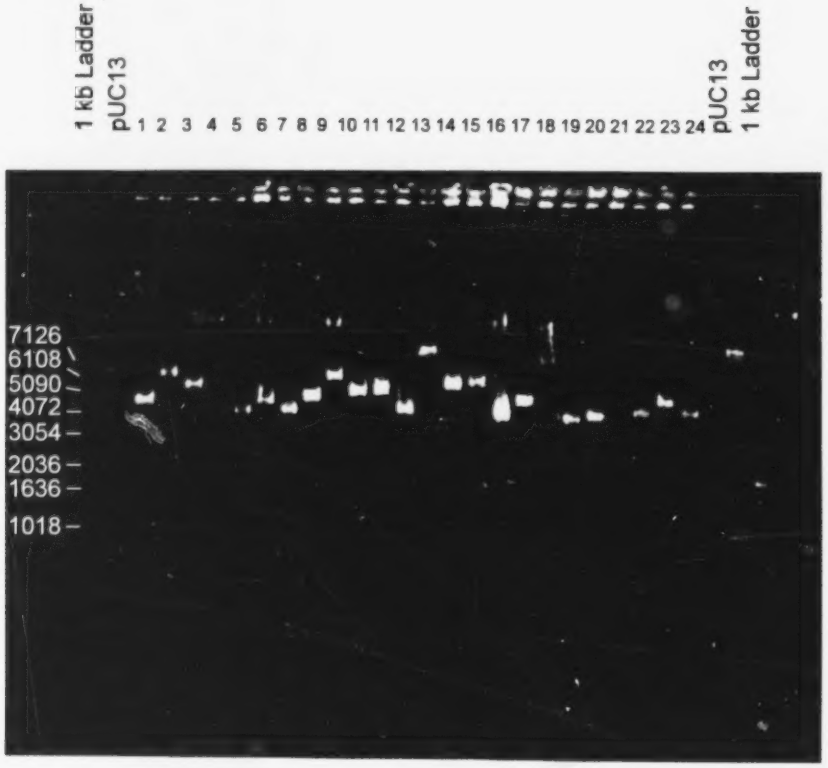

2
9
17 18
5.5
2.6

by restricting chromosomal DNA with *Sac*I, *Hpa*I or *Bgl*II and ligating to either *Sac*I, *Sma*I or *BamH*I cleaved pUC13 respectively. Both a forced ligation and shotgun strategy was employed for this purpose. In the former, digested chromosomal DNA was separated on an agarose gel and the regions corresponding to the size ranges for each of the restriction digests was excised from the gel. The purified DNA fragments were checked via Southern hybridization with the *gyrA* PCR probe and results indicated that the fragments isolated corresponded to *gyrA* DNA. For shotgun ligation, whole chromosmal DNA digests were ethanol preciptated to remove enzymes and excess salt, redissolved in TE buffer, and used directly for ligation. To reduce the amount of background *Hpa*I and *Sac*I-digested chromosomal DNA was first applied to a Chromaspin 1000 spin column to remove fragments 1.5 kb in size and smaller. 5 µl from each ligation reaction was used to transform competent DH10B *E. coli*. Several hundred white, carbenicillin-resistant colonies were selected and transferred to duplicate nylon membrane discs on LB agar supplemented with carbenicillin (500 µg/ml) plates. The filters were incubated overnight at 37^{0}C and one of each set was lysed, denatured, baked and then hybridised with a DIG-dUTP labelled *gyrA* probe. None of the membranes hybridised with the probe in either the forced or shotgun methods. The shotgun ligation was repeated under the same conditions and 24 transformants were selected at random and plasmid DNA prepared from these. There was a weak positive reaction after prolonged exposure of the hybridized blot to X-ray film (Figure 3.3B). Plasmid DNA was isolated from this weakly positive transformant and subsequent restrictions and hybridizations confirmed this was a *gyrA* clone. The initial clone, pEMP3, contained a 5.5 kb *Sac*I chromosomal fragment but

Figure 3.3. Sucloning of UA60 *gyrA*.

Electrophoretic analysis of plasmid minipreps of *C. fetus* UA60 *Sac*I fragment shotgun ligation to *Sac*I digested pUC13 (A) in 0.8% TAE agarose. A single lane hybridized with the UA60 *gyrA* PCR probe (B) which corresponds to sample 8 (lane 10).

A.

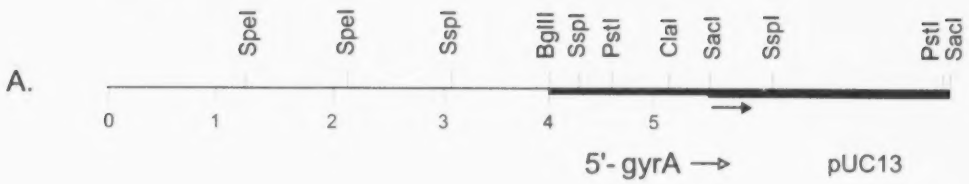

1 kb Ladder
pUC13
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
pUC13
1 kb Ladder
7126
6108
5090
4072
3054
2036
1636
1018

8

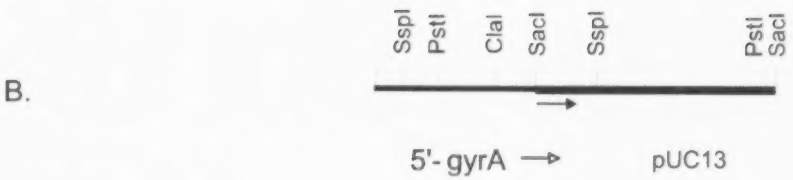
B.

Figure 3.4. Restriction maps of pEMP3 and derivative pEMP3.1.

The 5'-*gyrA* sequences are indicated with the thick solid black line. pUC13 is represented by the checkered bar. Sizes for pEMP3 are in kilobasepairs and the solid arrow indicates the direction of *lacZα* in pUC. The hollow arrow indicates the orientation of the cloned fragment.



pEMP3



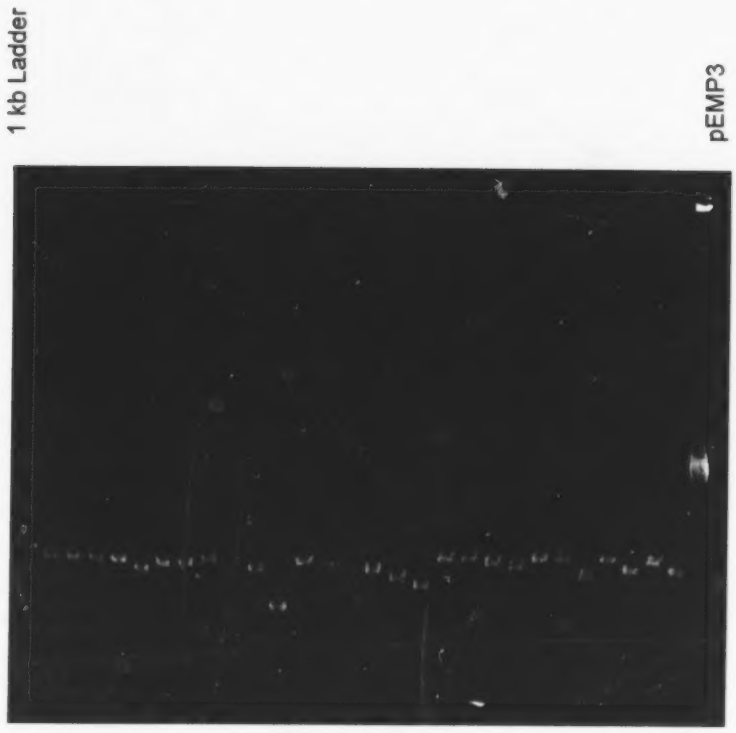
pEMP3.1

restriction mapping and hybridization with the *gyrA* probe indicated only a 1.4 kb *Bgl*II-*Sac*I fragment corresponded to the N-terminal fragment of the gene. This 1.4 kb fragment was further subcloned in pBluescript II SK (pEMP3.1) (Figure 3.4). Since the *gyrA* probe also hybridized to a 2.7 kb *Bgl*II UA60 fragment, attempts were made to subclone this. Three strategies were employed: (I) purifying the region from an agarose gel; (ii) shotgun ligation; and (iii) size-exclusion chromatography selection for fragments larger than 1.5 kb. All of these attempts and others using different enzymes failed. PCR was used to amplify a probe to identify the C-terminal fragment of the *gyrA* gene on a Southern blot. A forward primer was designed to the 3′ region of the N-terminal fragement in plasmid pEMP3. This oligonucleotide 60GyrA1, specific to nucleotides 1219 through 1237 in UA60, which is upstream of the *Sac*I site, had the sequence, 5′-TCCGCGTTGTTATAGAGCT-3′. A second oligonucleotide, 60GyrA2, was designed as reverse primer to the downstream *Bgl*II site from the C-terminal region of *C. jejuni gyrA*. This primer had the sequence, 5′-CTACGCTAATAAGATCT-3′, and is complementary to nucleotides 2663 through 2679 of UA60. The six nucleosides at the 3′ terminus of this primer comprise the *Bgl*II restriction site and except for the three residues at the 5′ terminus, are the only homologous bases to *C. fetus*. A 1.5 kb fragment was amplified with PCR. This fragment was directly sequenced using the PCR primers as cycle sequencing primers. Based on this preliminary sequence, the fragment was found to be about 70% homlogous to the C-terminal region of *C. jejuni gyrA*. The PCR product was randomly-labelled with [^{32}P]dCTP and used as probe against a Southern blot of UA60 chromosomal DNA digested with *Bgl*II and *Sac*I. A 1.7 kb *Sac*I fragment was identified as was the same 2.7 kb *Bgl*II fragment that hybridized with the N-terminal probe. The region from an agarose gel corresponding to the 1.7 kb *Sac*I band was excised, the DNA was purified, and ligated with *Sac*I digested pBluescript II SK.

Figure 3.5. Subcloning of the 3′ region of UA60 *gyrA*.

(A) PCR analysis of transformants from ligation of 1.6 kb to 2 kb fragments from *Sac*I-digested UA60 chromosomal DNA to *Sac*I-digested and dephosphorylated pBluescript II SK+. 5µl of a 50 µl volume were loaded onto 1.0% TAE agarose gels. (B) [^{32}P] in-gel hybridization of (A) using the 1.5 kb C-terminal *gyrA* PCR product as probe.

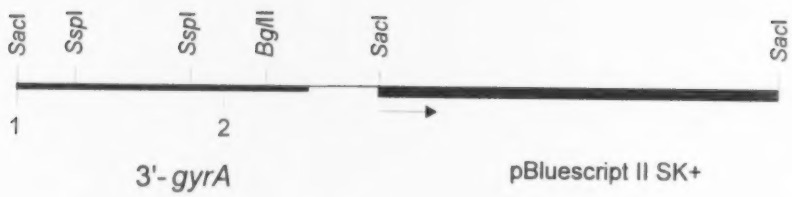
A.



B.

Figure 3.6. Restriction map of plasmid pEMP19a.

pEMP19a contains the 3′ region of the *C. fetus* UA60 *gyrA* gene. The thick solid black line represents the 3′-*gyrA* region, the thin line corresponds to the 3′ downstream flanking DNA and pBluescript II SK+ is represented by the checkered line. The arrow indicates the orientation of the *lacZ*α. Sizes are given in kilobasepairs.



pEMP19a

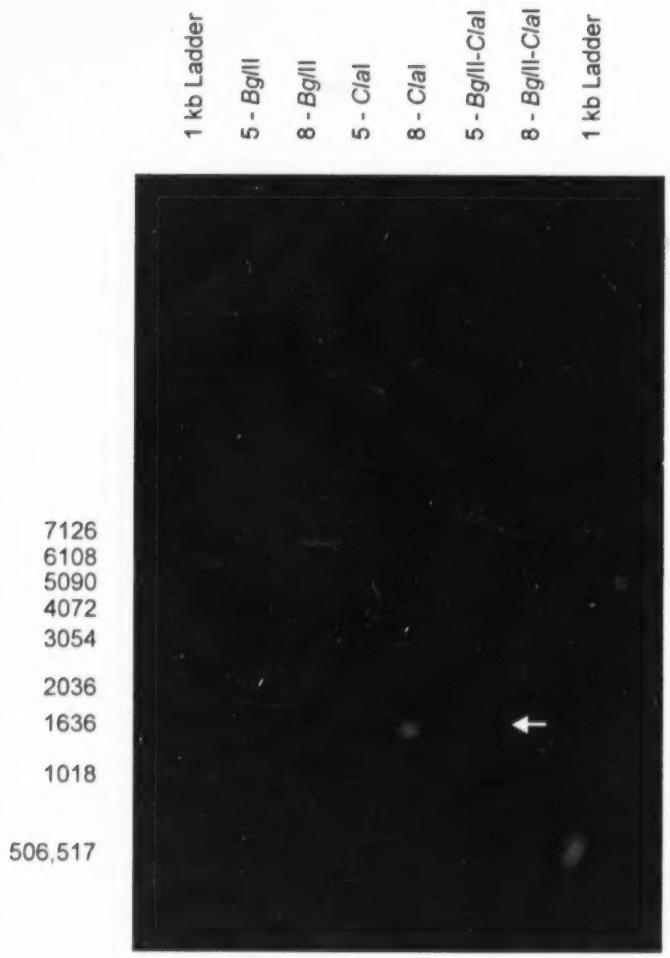
A total of 28 transformants were screened using PCR and 5 µl of each 50 µl reaction volume was loaded onto a 0.8% agarose gel (Figure 3.5A). After electrophoresis, the gel was dried and hybridised with the [^{32}P]-labelled C- terminal PCR probe (Figure 3.5B). 10 out of the 28 total recombinants analyzed were found to hybridize. Two of these, pEMP19a and pEMP19b, were selected at random and plasmid DNA isolated and restricted with *BamH*I and *Bgl*II. Plasmid pBluescript II SK possesses a single *BamH*I restriction site located within the multiple cloning region 40 bp upstream of the *Sac*I site. The 3′ *Sac*I *gyrA* fragment from UA60 has a single *Bgl*II restriction site approximately 1.2 kb from the 5′ terminus of the fragment. By digesting with both *BamH*I and *Bgl*II it will be possible to determine the orientation of the insert in these clones. Since the BamHI site is invariant in terms of location, ie., it is always 40 bp from the SacI insertion site, differences in restriction fragments sizes can only result from the insertion of the *gyrA* fragment in opposite orientations. It was discovered that these two plasmids had inserts in opposite orientations as pEMP19a yielded two fragments of approximately 3.3 kb and 1.2 kb in size (Figure 3.6) and clone pEMP19b yielded two fragments of about 4.1 kb and 500 bp. These recombinant plasmids were used for generating deletion mutants for sequencing.

3.2 Assembly of a contiguous *gyrA* clone

As the *gyrA* gene was originally cloned as two separate fragments, a 1.4 kb *Bgl*II-*Sac*I N-terminal fragment and a 1.7 kb C-terminal *Sac*I fragment, it was desirable to construct a single plasmid of the correct orientation. These separate inserts were maintained on pUC13 and pBluescript II SK vectors respectively. The N-terminal fragment was excised from pEMP3 with *Bgl*II and *Sac*I and ligated to *BamH*I and *Sac*I cut pBluescript

Figure 3.7. Analysis of plasmids from the construction of a complete UA60 *gyrA* gene.

Clones 5 and 8 were digested with *Bgl*II (lanes 2 and 3 respectively), *Cla*I (lanes 4 and 5 respectively) and double digested with both enzymes (lanes 6 and 7 respectively). The arrow indicates the fragment with the correct size of 1.5 kb from the *Bgl*II/*Cla*I double digest of clone 5 (pEMP20) which is expected from the ligation of fragments in the correct orientation.


1 kb Ladder
5 - BglII
8 - BglII
5 - ClaI
8 - ClaI
5 - BglII-ClaI
8 - BglII-ClaI
1 kb Ladder
7126
6108
5090
4072
3054
2036
1636
1018
506,517

II SK. The resulting plasmid, pEMP9, was then cut with *Sac*I to linearize it and treated with shrimp alkaline phosphatase to remove 5' phosphate groups to reduce background due to plasmid recircularizing during ligation. Linearized pEMP9 was ligated with the 1.5 kb C-terminal SacI fragment obtained from pEMP19a and 5 µl of the ligation reaction was used to transform $CaCl_2$-competent DH10B. Inserts were characterized with PCR and five samples appeared positive, as each amplified about a 3.0 kb product. Plasmid DNA from these transformants was isolated for further analysis. These PCR products were digested with *Bgl*II and *Cla*I and one sample (pEMP22) yielded the expected restriction pattern consistent with a contiguous *gyrA* gene of 3.4 kb, and 1.5 kb and 1.2 kb fragments respectively (Figure 3.7).

3.3 Polymerase chain reaction screening for inserts and deletions

This method was utilized for screening transformants for the presence of inserts as well as their sizes. It was also used to characterize exonuclease III deletions and to confirm the preservation of priming sites for sequencing. Single colonies were picked from plates containing transformants after overnight incubation. The cells were first transferred to an appropriately labelled master and then to a 0.5 ml microcentrifuge tube containing 50 µl of PCR mix. The reactions were carried out as normal using an annealing temperature of 50^0C with the exception of the inclusion of a 5 minute 'hot start' at 95^0C to lyse the cells and denature chromosomal DNA prior to beginning the amplification program. No differences were observed in amplification whether *Taq* polymerase was added prior to the 'hot start' or after. Universal pUC/M13 forward and reverse primers and T3 and T7 primers were used for amplification of the multiple cloning site including inserts. False positive

colonies, ie., those that appeared white on X-gal + IPTG agar using β-galactosidase selection, yielded amplification products of around 159 bp from pBluescript SK II using T3 and T7 primers. Products of up to 5 kb were amplified using this method and an extension time of 2.5 minutes at 72^{0}C. Typical results are similar to those shown in Figure 3.5 in the analysis of C-terminal *gyrA* transformants of UA60. Those samples that appeared positive by this method were then grown up in larger scale and plasmid DNA prepared for sequencing or other analysis.

3.4 Selecting *in vitro* ciprofloxacin-resistant *C. fetus*

UA60 was grown on BHI agar overnight and this culture was used to inoculate an overnight broth culture in BHI broth enriched with 5% serum. For the initial selection, 50 µl of the overnight culture was spread onto MH agar supplemented with 0.125 µg/ml ciprofloxacin. Small colonies were visible after 3 days growth at 37^{0}C. Single colonies were further subcultured on exponentially increasing amounts of ciprofloxacin. Subsequent to the initial selection, growth was visible after 24 h at each concentration of fluoroquinolone. The maximum level of ciprofloxacin on which UA60R8 could be cultured was 8 µg/ml (Table 3.1).

3.5 *gyrA* sequence analysis

A major ORF from nucleotides 311 to 2896 was found by computer sequence analysis using the program FRAMES from the Wisconsin GCG software package. The putative start site for the amino acid sequence was determined on the basis of protein sequence homology with other GyrA proteins. The *gyrA* gene is 2586 bp in length and

Table 3.1 MICs of nalidixic acid and ciprofloxacin for *Campylobacters.*

Strain	Nalidixic Acid (μg/ml)	Ciprofloxacin (μg/ml)
C. fetus UA60[a]	256	<0.125
C. fetus UA1221[b]	512	16
C. coli UA585[c]	4	<0.125
C. coli UA417[d]	16	<0.125
C. fetus UA60R8[e]	256	8

a. ATCC 27374 from The Hospital for Sick Children, Toronto
b. Cip^R clinical isolate from LCDC (LCDC# 15-515)
c. Strain# 118114 from Public Health Lab Service, Wales
d. Nal^R strain from LCDC (LCDC# C2633)
e. Cip^R resistant lab mutant

Figure 3.8. Nucleotide sequence of the complete UA60 *gyrA* gene.

Nucleotide sequence of the *C. fetus* subsp. *fetus* UA60 *gyrA* gene (GenBank accession number u25640). The deduced amino acid sequence starting at nucleotide 311 and ending at nucleotide 2896 is included beneath the sequence. The start (ATG) and stop (TAA) codons identified by amino acid sequence homology are highlighted in bold type. The putative -10 region and the ribosome binding site (SD) are underlined and labeled above these sites. Restriction sites are underlined and indicated below the recognition sequences. Primer sites for GyrA1, GyrA2, 60GyrA1, and 60GyrA2 are shown above the sequence with the direction of the arrow indicating the orientation of the oligonucleotide.

```
          10                                                  50
CGCTCTAGAACTAGAGGATCTTCCTAAGAATAGCGATTTAAGTGTAGATAAAATAGTAAA
  XbaI

          70                                                  110
TTTATTACAAAAATCCGGTAATTTTTTAGAATGCGAGTTCGGATCTTCTACTATAACTGC

          130                                                 170
GATTAACGCAAAAGACTATTTTAACGAAGTTCTAAAAGTTTTAAAAATAAATGAAACCTC

          190                                                 230
GCTTTTAAAACAGTAAAAGCTTATTTTGATTTTTTTATGCAAATAATGTTTGTAAATTTA
                HindIII

          250                                                 290     -10
AAAATTGTCTATTTAGCAAGTTTTTATCAAATTTTAGCTACAATCGTTGTAGCTATTTTT

   SD     310                                                 350
AAGGATAAAAATGGAAGAAAATATTTTCAGTTCAAATCAAGATATCGACGCTATAGACGT
          M  E  E  N  I  F  S  S  N  Q  D  I  D  A  I  D  V
                SspI                       EcoRV

          370                                                 410
TGAAGACTCTATAAAAGCAAGCTACCTAGATTACTCTATGAGCGTTATAATAGGTCGTGC
 E  D  S  I  K  A  S  Y  L  D  Y  S  M  S  V  I  I  G  R  A

                GyrA1
          430 ------------------------------------------>     470
TTTGCCAGATGCAAGAGACGGTTTAAAACCGGTTCATCGTCGCATACTTTATGCTATGAA
 L  P  D  A  R  D  G  L  K  P  V  H  R  R  I  L  Y  A  M  N

          490                                                 530
CGATCTTGGCGTAGGTAGTCGCAGCCCATATAAAAAGTCTGCTCGTATAGTAGGTGATGT
 D  L  G  V  G  S  R  S  P  Y  K  K  S  A  R  I  V  G  D  V

          550                                                 590
TATCGGTAAGTATCACCCGCACGGCGATACTGCGGTATATGACGCTTTAGTTAGAATGGC
 I  G  K  Y  H  P  H  G  D  T  A  V  Y  D  A  L  V  R  M  A

          610                                                 650
TCAGAACTTTTCTATGAGAGTTCCTGCAGTAGATGGTCAAGGAAACTTTGGCTCAGTCGA
 Q  N  F  S  M  R  V  P  A  V  D  G  Q  G  N  F  G  S  V  D
                PstI
```

670 710

TGGCGATGGCGCAGCCGCTATGCGTTATACTGAAGCTAGAATGACGGTTTTGGCAGAGGA

G D G A A A M R Y T E A R M T V L A E E

730 770

ACTTTTAAGAGATTTAGATAAAGATACGGTTGATTTTATACCAAATTATGATGATAGTTT

L L R D L D K D T V D F I P N Y D D S L

790 830

AAGCGAACCAGATGTTTTACCCGCGCGCGTACCGAATTTGTTGTTAAATGGATCGAGCGG

S E P D V L P A R V P N L L L N G S S G

GyrA2

850 890

TATCGCTGTTGGTATGGCGACAAATATCCCTCCACATAGTTTAGATGAGCTAGTAAATGG

I A V G M A T N I P P H S L D E L V N G

910 950

ATTACTCACTCTTTTAGACGATAAAGAAGTTGGTTTAGAGGATATTATGACTCATATAAA

L L T L L D D K E V G L E D I M T H I K

970 1010

GGGTCCTGATTTTCCAACCGGCGGTATAATTTTTGGGAAAAAAGGTATTATCGAAGCTTA

G P D F P T G G I I F G K K G I I E A Y

HindIII

1030 1070

TAAAACAGGTCGAGGACGTATCAAACTAAGAGCTAAAACTCATATTGAAAAAAAACCAAA

K T G R G R I K L R A K T H I E K K P N

1090 1130

TAAAGATGTTATAGTAGTCGATGAACTTCCATATCAAGTAAATAAAGCCAAGCTTCATGC

K D V I V V D E L P Y Q V N K A K L H A

HindIII

1150 1190

AGATATAGCCGATCTTGTAAAAGAGAAGCTCATCGATGGTATAAGCGAAGTAAGGGATGA

D I A D L V K E K L I D G I S E V R D E

ClaI

60GyrA1

1210 1250

GAGCGATAGAGACGGAATTCGTCTTGTTATAGAGCTAAAACGCGATGCTATGAGTGAGAT

S D R D G I R L V I E L K R D A M S E I

EcoRI

1270 1310
CGTGTTAAATAATTTATTTAAATCTACTCAAATGGAAGTTACTTTCGGCGTTATAATGCT
V L N N L F K S T Q M E V T F G V I M L

1330 1370
TGCTATAAATAATAAAGAGCCAAAAGTATTTTCTCTTTTGGAGCTTTTAAAGCTGTTTTT
A I N N K E P K V F S L L E L L K L F L

1390 1430
AAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGC
N H R K T V I I R R T I F E L Q K A R A

1450 1490
AAGAGCTCATATTTTAGAAGGTTTAAAAATAGCGCTTGATAATATAGACGCGGTTATCAA
R A H I L E G L K I A L D N I D A V I N
SacI

1510 1550
TCTGATAAAAACCAGCGCCGATACAAACTCTGCAAGAGACGGTTTGATGGCTAAATTCGG
L I K T S A D T N S A R D G L M A K F G

1570 1610
ACTTTCTGAACTTCAAAGCAATGCTATTCTTGATATGAGGCTTAGTAAGTTAACAGGACT
L S E L Q S N A I L D M R L S K L T G L
HincII

1630 1670
TGAGAGAGAAAAATTAGAAGCCGAACTAAAAGAGATTTTAGAGCTTATAGAAAAACTAGA
E R E K L E A E L K E I L E L I E K L D

1690 1730
CGCAATATTAAAAAGCGAAACTTTGATAGAAAATATAATAAGAGACGAGCTTTTAGAAAT
A I L K S E T L I E N I I R D E L L E I
SspI

1750 1790
CAAATCTAAATTTAAATGTCCGCGCATCACCGACATAGTCGATGATTATGATGATATAGA
K S K F K C P R I T D I V D D Y D D I D

1810 1850
CGTAGAAGACTTGATACCAAATGAAAATATGGTAGTTACCATAACTCACCGCGGATATAT
V E D L I P N E N M V V T I T H R G Y I
SacII

1870 1910
AAAACGCGTTCCTAGTAAGAGCTATGAAAAGCAAAAACGCGGTGGTAAAGGCAAGGTTGC
K R V P S K S Y E K Q K R G G K G K V A

1930 1970
AGTAACTACGTATGATGATGATTTTATAGAGAGTTTCTTTACTTGCATGAGTCATGATAC
V T T Y D D D F I E S F F T C M S H D T

1990 2030
GCTTATGTTTGTGACTGATCGCGGTCAGCTTTACTGGCTTAAAGTTTATAAAATTCCAGA
L M F V T D R G Q L Y W L K V Y K I P E

2050 2090
AGGCAGCCGAACCGCAAAGGGAAAAGCGGTTGTAAATCTTATATCGCTTCAAGCAGACGA
G S R T A K G K A V V N L I S L Q A D E

2110 2150
AAAGATAAAAGCTATCATACCTACAACGGATTTTGATGAGAGCAAATCTTTAGCTTTCTT
K I K A I I P T T D F D E S K S L A F F

2170 2210
CACTAAAAACGGTATAGTAAAACGTACGAATTTAAGTGAGTTTAAAAATATTCGTTCAAT
T K N G I V K R T N L S E F K N I R S I

SspI

2230 2270
AGGCGTGAAAGCTATAAATTTAGATGATAATGATGAGCTTGTGACTGTTGTAATCGCTAA
G V K A I N L D D N D E L V T V V I A N

2290 2330
TAGCGAGCCTGATGAGAGCTATGATGATAGCTTTGAAGATGGCGAAGGTGTTTCAAATTT
S E P D E S Y D D S F E D G E G V S N L

2350 2390
GCAAACCATAAGCGAAGATAACTCCGAAAACAGTCTTGAGAGCGGAAAAATGCTATTTGC
Q T I S E D N S E N S L E S G K M L F A

2410 2450
TGTTACTAAAAAAGGTATGTGTATCAAATTTGCTTTAAATAAAGTAAGACAGATAGGAAG
V T K K G M C I K F A L N K V R Q I G R

2470 2510
AGTTAGCCGTGGCGTAACCGCTATAAGATTTAAAGAGAATTTAGATGAAGTTGTTGGAGC
V S R G V T A I R F K E N L D E V V G A

2530 2570
AGTCGTTATAGAAAATGATTCTCAAGAAATTTTAAGCGTGAGCCAAAAAGGTATAGGAAA
V V I E N D S Q E I L S V S Q K G I G K

2590 2630
ACGCACAACGGCTGATGAGTATAGATTGCAAAGTCGCGGAGGTAAAGGCGTCATTTGTAT
R T T A D E Y R L Q S R G G K G V I C M

60GyrA2

2650 2690
GAAATTAACTCCAAAAACAAAAGATCTTGTTGGTGTAGTAATGGTAGATGAAGAGATGGA
K L T P K T K D L V G V V M V D E E M D

BglII

2710 2750
TCTTATGGCGCTAACATCAAGCGGCAAGATGATAAGAGTAGATATGCAAAGCATCAGAAA
L M A L T S S G K M I R V D M Q S I R K

2770 2810
AGCAGGGCGTAATACAAGCGGAGTCATAGTCGTAAATGTAGATGGCGATGAGGTTGTAAG
A G R N T S G V I V V N V D G D E V V S

2830 2870
TATAGCAAGATGCCCTAAAGAAGAGAGCGATGATGACGATATTGTTGCAGATGATACTCA
I A R C P K E E S D D D D I V A D D T Q

2890 2930
AGAACAAGATATGGAA**TAA**GTTTTGCTTAGAATACATAATTTAAATTTAAGGTAATAAAT
E Q D M E

2950 2990
ATGAAATCATTAATTTTTGCATTACTAGTAGTCGGTGTTTTCGTAGGTTGCGCCGCATTT

3010 3050
ACTCCAGAGCCTAAAACACAAGATGTTGTGGTTCAAAAAGTAGATAAAGACGACCTAAGA

3070 3110

GAAATAATCAAAAACGATAAAATGTTAGCCGGTATGGAGATAAATCCAGAGGCTATTTTT

3130 3150

TCAGCAGTCGGCGAGGGAATTCCTCCTATAGATACAGTTAGTC

EcoRI

Figure 3.9. Amino acid sequence alignment of GyrA proteins.

C. fetus subsp. *fetus* UA60 (accession number u25640) protein sequence is compared to other GyrA proteins. The consensus sequence is indicated in bold type below the alignment. The grey box outlines the region shared by *C. fetus*, *C. jejuni*, *H. pylori*, and *N. gonorrhoeae*. *C. jejuni* (accession number a48902) was obtained from the PIR database; *B. subtilis* (accession number p05653), *E. coli* (accession number p09097), *K. pneumoniae* (accession number p14829), and *S. aureus* (accession number p20831) sequences were obtained from the SWISS-PROT database; and *E. carotova* (accession number ecgyrag), *H. pylori* (accession number l29481), and *N. gonorrhoeae* (accession number u08817) were translated from nucleotide sequences obtained from the the GenBank database. This alignment was performed with the PILEUP program of the Wisconsin GCG Software Package for UNIX (Genetics Computer Group, 1991).

```
C.fetus          MEENIFSS....NQDIDAIDVEDSIKASYLDYSMSVIIGRALPDARDGLKPV   48
C.jejuni         M.ENIFSK....DSDIELVDIENSIKSSYLDYSMSVIIGRALPDARDGLKPV   47
H.pylori         MQDRLVNE....TKNIVEVGIDSSIEESYLAYSMSVIIGRALPDARDGLKPV   48
B.subtilis       MSEQNTPQ....VR...EINISQEMRTSFLDYAMSVIVSRALPDVRDGLKPV   45
S.aureus         MAE..LPQ....SR.INERNITSEMRESFLDYAMSVIVARALPDVRDGLKPV   45
E.coli           MSD........LAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPV   44
K.pneumoniae     MSD........LAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPV   44
E.carotovora     MSD........LAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPV   44
P.aeruginosa     MGE........LAKEILPVNIEDELKQSYLDYAMSVIVGRALPDARDGLKPV   44
N.gonorrhoeae    MTDATIRHDHKFALETLPVSLEDEMRKSYLDYAMSVIVGRALPDVRDGLKPV   52
Consensus        M-------------------------SYL-Y-MSVIV-RALPD-RDGLKPV   52

HRRILYAM.NDLGVGSRSPYKKSARIVGDVIGKYHPHGDTAVYDALVRMAQNFSMRVPAVDGQGNFGSVD  117
HRRILYAMQND.EAKSRTDFVKSARIVGAVIGRYHPHGDTAVYDALVRMAQDFSMRYPSITGQGNFGSID  116
HRRILYAM.HELGLTSKVAYKKSARIVGDVIGKYHPHGDNAVYDALVRMAQDFSMRLELVDGQGNFGSID  117
HRRILYAM.NDLGMTSDKPYKKSARIVGEVIGKYHPHGDSAVYESMVRMAQDFNYRYMLVDGHGNFGSVD  114
HRRILYGL.NEQGMTPDKSYKKSARIVGDVMGKYHPHGDSSIYEAMVRMAQDFSYRYPLVDGQGNFGSMD  114
HRRVLYAM.NVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQGNFGSID  113
HRRVLYAM.NVLGNDWNKAYKKSARVVGDVIGKYHPHGDTAVYDTIVRMAQPFSLRYMLVDGQGNFGSVD  113
HRRVLYAM.SVLGNDWNKPYKKSARVVGDVIGKYHPHGDSAVYETIVRMAQPFSLRYMLVDGQGNFGSID  113
HRRVLYAM.SELGNDWNKPYKKSARVVGDVIGKYHPHGDTAVYDTIVRMAQPFSLRYMLVDGQGNFGSVD  113
HRRVLYAM.HELKNNWNAAYKKSARIVGDVIGKYHPHGDSAVYDTIVRMAQNFAMRYVLIDGQGNFGSVD  121
HRR-LY-M-----------Y-KSARIVG-V-G-YHPHGD--VYD--VRMAQ-F--R---V-G-GNFGS-D  122

GDGAAAMRYTEARMTVLAEELLRDLDKDTVDFIPNYDDSLSEPDVLPARVPNLLLNGSSGIAVGMATNIP  187
GDSAAAMRYTEAKMSKLSHELLKDIDKDTVDFVPNYDGSESEPDVLPSRVPNLLLNGSSGIAVGMATNIP  186
GDNAAAMRYTEARMTKASEEILRDIDKDTIDFVPNYDDTLKEPDILPSRLPNLLVNGANGIAVGMATSIP  187
GDSAAAMRYTEARMSKISMEILRDITKDTIDYQDNYDGSEREPVVMPSRFPNLLVNGAAGIAVGMATNIP  184
GDGAAAMRYTEARMTKITLELLRDINKDTIDFIDNYDGNEREPSVLPARFPNLLANGASGIAVGMATNIP  184
GDSAAAMRYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIP  183
GDSAAAMRYTEIRMSKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSFGIAVGMATNIP  183
GDSAAAMRYTEIRMSKIAHELLADLEKETVDFVPNYDGTEQIPDVMPTRIPNLLVNGSSGIAVGMATNIP  183
GDNAAAMRYTEVRMAKLAHELLADLEKETVDWVPNYDGTEQIPAVMPTKIPNLLVNGSSGIAVGMATNIP  183
GLAAAAMRYTEIRMAKISHEMLADIEEETVNFGPNYDGSEHEPLVLPTRFPTLLVNGSSGIAVGMATNIP  191
G--AAAMRYTE--M-----E-L-D----TV-F--NYD-----P-V-P---P-LL-NG--GIAVGMAT-IP  192

PHSLDELVNGLLTLLDDKEVGLEDIMTHIKGPDFPTGGIIFGKKGIIEAYKTGRGRIKLRAKTHIE...K  254
PHSLNELIDGLLYLLDNKDASLEEIMQFIKGPDFPTGGIIYGKKGIIEAYRTGRGRVKVRAKTHIE...K  253
PHRIDEIIDALAHVLENPNAELDEILEFVKGPDFPTGGIIYGKAGIIEAYKTGRGRVKVRAKVHVE...K  254
PHQLGEIIDGVLAVSENPDITIPELMEVIPGPDFPTAGQILGRSGIRKAYESGRGSITIRAKAEIE..QT  252
PHNLTELINGVLSLSKNPDISIAELMEDIEGPDFPTAGLILGKSGIRRAYETGRGSIQMRSRAVIE..ER  252
PHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYIRARAEVEVDAK  253
PHNLTEVINGRLAYVEDEEISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYICARAEVEADAK  253
PHNLTEVVNGCLAYIDDENISLEGLMEHIKGPDFPTAAIINGRRGIEEAYRTGRGKIYIRARAEVEADAK  253
PHNLGEVIDGCLALMDNPDLTVDELMQYIPGPDFPTAGIINGRAGIIEAYRTGRGRIYIRARAVVEEMEK  253
PHNLTDTINACLRLLDEPKTEIDELIDIIQAPDFPTGATIYGLGGVREGYKTGRGRVVMRGKTHIEPIGK  261
PH---E-I--------------------I--PDFPT---I-G--GI---Y--GRG----------E----  262

KPNKDVIVVDELPYQVNKAKLHADIADLVKEKLIDGISEVRDE.SDRDGIRLVIELKRDAMSEIVLNNLF  323
KTNKDVIVIDELPYQTNKARLIEQIAELVKERQIEGISEVRDE.SNKEGIRVVIELKREAMSEIVLNNLF  322
TKNKEIIVLDEMPFQTNKAKLVEQISDLAREKQIEGISEVRDE.SDREGIRVVIELKRDAMSEIVLNHLY  323
SSGKERIIVTELPYQVNKAKLIEKIADLVRDKKIEGITDLRDE.SDRTGMRIVIEIRRDANANVILNNLY  321
GGGRQRIVVTEIPFQVNKARMIEKIAEVRDKKIDGITDLRDETSLRTGVRVVIDVRKDANASVILNNLLY  322
.TGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDE.SDKDGMRIVIEVKRDAVGEVVLNNLY  321
.TGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDE.SDKDGMRIVIEVKRDAVGRVVLNNLY  321
.TGRETIIVHEIPYQVNKARLIEKIAELVKDKRIEGISALRDE.SDKDGMRIVIEIKRDAVGEVVLNNLY  321
GGGREQIIITELPYQLNKARLIEKIAELVKEKKIEGISELRDE.SDKDGMRVVIELRRGEVGEVVLNNLY  322
NGERERIVIDEIPYQVNKAKLVEKIGDLVREKTLEGISELRDE.SDKSGMRVVIELKRNENAEVVLNQLY  330
------I---E-PYQ-NKA-L---I-EL--E----GI---RDE-S---G-R-VIE--------VVLN-LY  332
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```
KSTQMEVTFGVIMLAINNKEPKVFSLLELLKLFLNHRKTVIIRRTIFELQKARARAHILEGLKIALDNID  393
KSTTMESTFGVIMLAIHNKEPKIFSLLELLNLFLTHRKTVIIRRTIFELQKARARAHILEGLKIALDNID  392
KLTTMETTFSIILLAIYNKEPKIFTLLELLRLFLNHRKTIIIRRTIFELEKAKARAHILEGYLIALDNID  393
KQTALQTSFGINLLALVDGQPKVLTLKQCLEHYLDHQKVVIRRRTAYELRKAEARAHILEGLRVALDHLD  391
KQTPLQTSFGVNMIALVNGRPKLINLKEALVHYLEHQKTVVRRRTQYNLRKAKDRAHILEGLRIALDHID  392
SQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRREVVTRRTIFELRKARDRAHILEALAVALANID  391
SQTQLQVSFGINMVALHHGQPKIMNLKEIIAAFVRHRREVVTRRTILALRKARDRADILEALSIALANID  391
SQTQLQTSFGINMVALHQGQPKIMPLKDILVAFVRHRREVVTRRTIFELRKARDRAHILEGLAIALANID  391
AQTQLQSVFGINVVALVDGQPRTLNLKDMLEVFVRHRREVVTRRTVYELRKARERGHILEGQAVALSNID  392
KLTPLQDSFGINMVVLVDGQPRLLNLKQILSEFLRHRREVVTRRTLFRLKKARHEGHIAERKAVALSNID  400
--T-L---F-I---------P----L------F--H---VV-RRT-F-L-KA-----I-E---IAL---D  402

AVINLIKTSADTNSARDGLMA.....................................KFGLSELQSNAIL  427
EVIALIKNSSDNNTARDSLVA.....................................KFGLSELQANAIL  426
EIVQLIKTSPSPEAAKNALME.....................................RFTLSEIQSKAIL  427
AVISLIRNSQTAEIARTGLI.......................................EQFSLTEKQAQAIL  425
EIISTIRESDTDKVAMESLQ.......................................QRFKLSEKQAQAIL  426
PIIELIRHAPTPAEAKTALVANPWQLGNVAAMLERAGDDAA..RPEWLEPEFGVRDGLYYLTEQQAQAIL  459
PIIELIRRAPTPAEAKAGLIARSWDLGNVSAMLE.AGDDAA..RPEWLEPEFGVRDGQYYLTEQQAQAIL  458
PIIELIRRAASPAEAKASLIAQAWELGSVATMLERAGDDAA..RPEWLEPEFGIRDGRYYLTEQQAQAIL  459
PVIELIKSSPTPAEAKERLIATAWESSAVEAMVERAGADAC..RPEDLDPQYGLRDGKYYLSPEQAQAIL  460
EIIKLIKESPNAAEAKEKLLARPWASSLVEEMLTRSGLDLEMMRPEGLVANIGLKKQGYYLSEIQADAIL  470
-II--I--------A---L-------------------------------------------L---Q--AIL  472

DMRLSKLTGLEREKLEAELKEILELIEKLDAILKSETLIENIIRDELLEIKSKFKCPRITDIV.DDYDDI  496
DMKLGRLTGLEREKIENELAELMKEIARLEEILKSETLLENLIRDELKEIRSKFDVPRITQIE.DDYDDI  495
EMRLQRLTGLERDKIKEEYQNLLELIDDLNGILKSEDRLNGVVKTELLEVKEQFSSPRRTEIQ.ESYESI  496
DMRLQRLTGLEREKIEEEYQSLVKLIAELKDILANEYKVLEIIREELTEIKERFNDERRTEIVTSGLETI  495
DMRLRRLTGLERDKIEAEYNELLNYISELEAILADEEVLLQLVRDELTEIRDRFGDDRRTEIQLGGFEDL  496
DLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEITANS.ADI  528
DLRLQKLTGLEHEKLLDEYKELLEQIAELLHILGSADRLMEVIREELELVREQFGDARRTDITANS.VDI  527
DLRLQKLTGMEHEKLLDEYKELLAEIAELLYILNSPERLMEVIREELEAFKTQYSDERRTEITANT.ADI  528
ELRLHRLTGLEHEKLLSEYQEILNLIGELIRILTNPARLMEVIREELEAVKAEFGDARRTEIVASQ.VDL  529
RMSLRNLTGLDQKEIIESYKNLMGKIIDFVDILSKPERITQIIRDELEEIKTNYGDERRSEINPFG.GDI  539
-M-L--LTGLE--------------I--L--IL---------I--EL------F---R---I--------  542

DVEDLIPNENMVVTITHRGYIKRVPSKSYEKQKRGGKGKVAVTTYDDDFIESFFTCMSHDTLMFVTDRGQ  566
DIEDLIPNENMVVTITHRGYIKRVPSKQYEKQKRGGKGKLAVTTYDDDFIESFFTANTHDTLMFVTDRGQ  565
DIEDLIANEPMVVSMSYKGYVKRVDLKVYEKQNRGGKGKLSGSTYEDDFIENFFVANTHDILLFITNKGQ  566
EDEDLIERENIVVTLTHNGYVKRLPASTYRSQKRGGKGVQGMGTNEDDFVEHLISTSTHDTILFFSNKGK  565
EDEDLIPEEQIVITLSHNNYIKRLPVSTYRAQNRGGRGVQGMNTLEEDFVSQLVTLSTHDHVLFFTNKGR  566
NLEDLITQEDVVVTLSHQGYVKYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGR  598
NIEDLITQEDVVVTLSHEGYVKYQPVNDYEAQRRGGKGKSAPRIKEEDFIDRLLVANTHDTILCFSSRGR  597
NIEDLINQEDVVVTLSHQGYVKYQPLSDYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDTILCFSSRGR  598
TIADLITEEDRVVTISHGGYAKSQPLAAYQAQRRGGKGKSATGMKDEDYIEHLLVANSHATLLLFSSKGK  599
ADEDLIPQREMVVTLTHGGYIKTQPTTDYQAQRRGGRGKQAAATKDEDFIETLFVANTHDYLMCFTNLGK  609
---DLI-----VV------Y-K------Y--Q-RGG-G-------EEDFI--L-----H---L-----G-  612

LYWLKVYKIPEGSRTAKGKAVVNLISLQADEKIKAIIPTT.....................................  606
LYWLKVYKIPEGSRTAKGKAVVNLINLQAEEKIMAIIPTT.....................................  605
LYHLKVYKIPEASRIAMGKAIVNLISLAPDEKIMATLSTK.....................................  606
VYRAKGYEIPEYGRTAKGIPIINLLEVEKGEWINAIIPVT.....................................  605
VYKLKGYEVPELSRQSKGIPVVNAIELENDEVISTMIAVK.....................................  606
VYSMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVT.....................................  638
LYWMKVYQVPEASRGARGRPIVNLLPLEANERYTAILPVR.....................................  637
LYWMKVYQLPEASRGARGRPIVNLLPLEADERITAILPVR.....................................  638
VYWLRTFEIPEASRTARGRPLVNLLPLDEGERITAMLQIDLEALQQNGGADDDLDEAEGAVLEGEVVEAA  669
CHWIKVYKLPEGGRNSRGRPINNVIQLEEGEKVSAILAVR.....................................  649
------Y--PE--R---G----N-------E---------------------------------------  682
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DFDES.....KSLAFF..TKNDIVKRTNLSEFKNIRSIGVKAINLDDNDELVTVVIANSEPDE  662
DFDES.....KSLCFF..TKNDIVKRTNLSEYQNIRSVGVRAINLDENDELVTAIIVQRDEDE  661
DFSNE.....RSLAFF..TKNGVVKRTNLSEFGSNRSCGIRAIVLDEGDELVSAKVV......  656
EFNAE.....LYLFFTTKHGVSKRTSLSQFANIRNNGLIALSLREDDELMGVRLT.DGTKQ  660
DLESE.....DNFLVFATKRGVVKRSALSNFSRINRNGKIAISFREDDELIAVRLT.SGQED  662
EFE.E.....GVKVFMATANGTVKKTVLTEFNRLRTAGKVAIKLVDGDELIGVDLT.SGEDE  693
EYE.E.....GVNVFMATASGTVKKTPADEFSRPRSAGIIAVNLNEGDELIGVDLT.SGQDE  692
EYE.E.....GRHIFMATASGTVKKTALTEFSR.HVSGIIAVNLNEGDELIGVDLT.DGSDE  692
EVE.EVEGETAELVAEPTGAYIFMATAFGTVKKTPLVQFSRPRSSGLIALKLEEGDTLIAAAIT.DGAKE  737
EFP.E.....DQYVFFATAQGMVKKVQLSAFKNVRAQGIKAIALKEGDYLVGAAQT.GGADD  704
E----------TG--K------F------G--A--L-E-D-L----------  752

SY....DDSFEDGEGVSNLQTISEDNSENSLES....GK.MLFAVTKKGMCIKFALNKVRQIGRVSRGVT  723
IFATGGEENLENQE.IENLD...DENLENE.ESVSTQGK.MLFAVTKKGMCIKFPLAKVREIGRVSRGVT  725
.....DKN.....AKHLLIA.SHLGIFIKFPLEDVREMGRNARGVI  691
IIIGTKNGLLIR...FPE.....TDVREMGRTAAGVK  690
ILIGTSHASLIR...FPE.....STLRPLGRTATGVK  691
VMLFSAEGKVVR...FKE.....SSVRAMGCNTTGVR  722
VMLFSAAGKVVR...FKE.....DDVRAMGRTATGVR  721
AMLFSAEGKVVR...FSE.....QAVRSMGRTATGVR  721
VMLFSSAGKVIR...FAE.....SVVRIMGRNARGVR  766
IMLFSNLGKAIR...FNEYWEKSGNDEAEDADIETEISDDLEDETADNENTLPSGKNGVRPSGRGSGGVR  771
----------R--G----G--  822

AIRFKENLDEVVGAVVI....ENDSQEILSVSQKGIGKRTTADEYRLQSRGGKGVICMKLT.PKTKDLVG  788
AIKFKEKNDELVGAVVI....ENDEQEILSISAKGIGKRTNAGEYRLQSRGGKGVICMKLT.EKTKDLIS  790
GIRLNEN.DFVVGAVVI....SDDGNKLLSVSENGLGKQTLAEAYREQSRGGKGVIGMKLT.QKTGNLVG  755
GITLTDD.DVVVGMEIL...EEES..HVLIVTEKGYGKRTPAEEYRTQSRGGKGLKTAKIT.ENNGQLVA  753
GITLREG.DEVVGLDVA...HENSVDEVLVVTENGYGKRTPVNDYRLSNRGGKGIKTATIT.ERNGNVVC  756
GIRLGEG.DKVVSLIV..PRGDG...AILTATQNGYGKRTAVAEYPTKSRATKGVISIKVT.ERNGLVVG  785
GIKLAGE.DKVVSLIV..PRGEG...RILTATENGYRKRTAVAEYPTKSRATQGVISIKVT.ERNGSVVG  784
GINLQGE.DRVVSLII..PRGEG...DILTVTQNGFGKRTAVSEYPTKSRATKGVISIKVS.ERNGKVVG  784
GMRLGKG.QQLISMLI..PESGA...QILTASERGFGKRTPLSKFPRRGRGGQGVIAM.VTNERNGALIA  833
GMRLPAD.GKIVSLITFAPETEESGLQVLTATANGYGKRTPIADYSRKNKGGQGSIAIN.TGERNGDLVA  839
---L-------V----------L-----G--K-T----Y--------G----------V-  892

VVMV.DEEMDLMALTSSGKMIRVDMQSIRKAGRNTSGVIVVNVDGDE.VVSIARCPKESDDDDIVADEDT  856
VVIV.DETMDLMALTSSGKMIRVDMQSIRKAGRNTSGVIVVNVENDE.VVSIAKCPKEENDEDE.LSDEN  857
VISVDDENLDLMILTASAKMIRVSIKDIRETGRNASGVKLIN.TADK.VMYVNSCPKEE..EPENLENSP  821
VKATKGEE.DLMIITASGVLIRMDINDISITGRVTQGVRLIRMAEEEHVATVALVEK....NEED..E.N  815
ITTVTGEE.DLMIVTNAGVIIRLDVADISQNGRAAQGVRLIRLGDDQFVSTVAKVKE....DAED..ETN  819
AVQVDDCD.QIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVAEP..VDEEDL.DT.  850
AVQVDDCD.QIMMITDAGTLVRIRVSEVSIVGRNTQGVILIRTAEDENVVALQRVAEP..VDDEEL.DA.  849
AVQVDAAD.QIMMITDAGTLVRTRVSEVSIVGRNTQGVTLIRTAEDEHVVGLQRVAEP..VEDEEL.DGV  850
AVQVQEGE.EIMLISDQGTLVRTRVDEVSLSGRNTQGVTLIKLASDEVLVGLERVQEPSGGDDEDLPEGE  902
ATLVGETD.DLMLITSGGVLIRTKVEQIRETGRAAAGVKLINLDEGETLVSLERVAE....DESELSGAS  904
----------M--------IR-----I---GR---GV--I----------  962

QEQDME.....  862
FGLDLQ.....  863
TQL.FE.....  826
EEEQ.....EEV.....  822
EDEQSTSTVSEDGTEQQREAVVNDETPGNAIHTEVIESEETDDDGRIEVRQDFMDRVEEDIQQSLDEDEE  889
..ID..GSAAEGDDEIAPEVDVDDE.PEEE.....  875
..ID..GSAAEGDEDIAPEADTDDDIAEDEE.....  876
VKVE..GEVAEDDDAID.DIDGDDDIAEDDE.....  878
EAAESLGESAESESEPAAEAEGNEE.....  922
V....ISNVTE.....PEAEN.....  916
----------  1032
```

both the *C. fetus* and *C. jejuni* GyrA's. There is no amino acid homology in this stretch among the three organisms. The *C. fetus* sequence is 34 amino acids in length, which is three residues shorter than found in *C. jejuni*. Of the 34 residues there are 12 negatively charged residues (Asp and Glu) and 9 Ser or Thr residues which suggests this region may be involved with hydrogen or electrostatic bonding. A putative Shine-Dalgarno sequence was found 4 bp upstream of the ORF as was a potential -10 region with the sequence TATTTT which is in good agreement with the *E. coli* consensus sequence of TATAAT (Figure 3.8). Similarly to *C. jejuni*, *E. coli* and *K. pneumoniae* promoters, a -35 region (TTGACA) was not identified. The QRDR in *C. fetus* extends from amino acid residues 71 to 110 or nucleotides 213 to 330. This amino acid sequence is similar to that of *C. jejuni* and *H. pylori*. Differences between *C. jejuni* and *C. fetus* are Ala-75/Asp (76), Arg-79/Lys (80), Asp-98/Asn (99), Tyr-103/Val (104), Ser-105/Ala (106), Ile-106/Val (107), Thr-108/Asp (109). The residues Ala-71, Thr-87 and Asp-91 in *C. fetus* are identical to the equivalent residues in *C. jejuni*. Mutations in these three positions, in particular, have been shown to be frequently associated with quinolone resistance in other bacterial gyrases including *C. jejuni*. Compared to other GyrA sequences these positions are highly conserved in *C. fetus*.

3.6 Sequence analysis of ciprofloxacin-resistant *C. fetus gyrA*

The QRDR in a ciprofloxacin-resistant *C. fetus* UA1221 as well as UA60R8 was amplified by PCR, blunt ends produced with T4 polymerase, and subcloned into *EcoR*V or *Hinc*II-cut pBluescript II SK+. The fragment was sequenced with both T3 and T7 primers with a cycle sequencing protocol and the nucleotide sequence compared with

Figure 3.10. Comparison of *C. fetus* and *C. jejuni* QRDRs in GyrA.

Alignment of the quinolone resistance determining region from *C. fetus* UA60 (Nal^R, Cip^S strain), *C. fetus* UA1221(Cip^R clinical isolate), *C. fetus* UA60R8 (Cip^R lab mutant), and *C. jejuni* UA580 (Nal^S). The first nucleotide in the triplet encoding amino acid residue 91 (residue 90 in *C. jejuni*) is underlined in each sequence (G in UA60 and UA580; T in UA1221 and UA60R8) and transversions are indicated in bold type as are the corresponding altered amino acid residues.

85 91

C. fetus (Nal^R/Cip^R) UA60 GGCGATACTGCGGTATATGACGCTTTA

G D T A V Y D A L

C. fetus (Nal^R/Cip^R) UA1221 GGCGATACTGCGGTATATTACGCTTTA

G D T A V Y **Y** A L

C. fetus (Nal^R/Cip^R) UA60R8 GGCGATACTGCGGTATATTACGCTTTA

G D T A V Y **Y** A L

84 90

C. jejuni (Nal^S) UA580 GGAGATACAGCAGTTTATGATGCTTTG

G D T A V Y D A L

the UA60 QRDR using the BESTFIT program of the GCG software. There was only one nucleotide difference identified between UA60 and UA1221 equivalent to position 581 of UA60; this was a G→T transversion. This corresponds to an amino acid change from Asp-91 to Tyr in the protein (Figure 3.10). Sequence analysis of the QRDR from UA60R8 also revealed a single amino acid substitution in this region. There was an identical G→T mutation resulting in the same Asp-91→Tyr change in this laboratory-derived mutant (Figure 3.10). These were the only differences detected within the region of the nucleotide sequence encoding the QRDR.

3.7 Construction of a chimeric *gyrA* gene

A hybrid *gyrA* gene was constructed using the sequences encoding the N-terminal region of *C. jejuni* UA580 *gyrA* and those encoding the C-terminal region of *C. fetus* UA60 *gyrA* (refer to Figure 3.11). Clone pYW75 (Figure 3.11), which contains the N-terminal fragment of the *C. jejuni gyrA* gene was digested with *EcoR*I. After electrophoresis of the digested DNA, a 3.7 kb fragment was excised from an agarose gel and purified. This 3.7 kb *EcoRI* UA580 fragment was ligated to *EcoR*I-digested pBluescript II SK+. The resultant plasmid was purified and restricted with *Sac*I to remove the 1.5 kb *Sac*I region downstream of the *gyrA* sequence, which includes the *EcoR*I site in the cloning vector, and religated to yield a plasmid with an insert 2.2 kb in size. This plasmid contained the 5′-region of the UA580 *gyrA* gene as well as approximately 800 bp of upstream flanking DNA. A *Sac*I fragment of about 1.7 kb in size containing the 3′ region of the UA60 *gyrA* gene was purified from pEMP19a. This 1.7 kb fragment ligated to the recombinant construct containing the 2.2 kb *C. jejuni* gyrA fragment. The ligation sample

Figure 3.11. Construction of chimeric *gyrA* plasmid pEMP22.

Plasmid pYW75 (A) (Used without permission from Wang *et al.*, 1993.), containing the 5'-*gyrA* region from *C. jejuni* UA580 was digested with *EcoR*I to yield a 3.7 kb fragment that was ligated to *EcoR*I cut vector pBluescript II SK+ (B). The 3'-gyrA region from *C. fetus* UA60 in plasmid pEMP19a (E) was digested with *Sac*I to yield a 1.7 kb fragment (D) that was ligated to *Sac*I digested (B). The resulting plasmid had a 3.9 kb insert (C) corresponding to the 1.7 kb *Sac*I fragment from pEMP19a and a 2.2 kb *EcoR*I-*Sac*I fragment from pYW75.

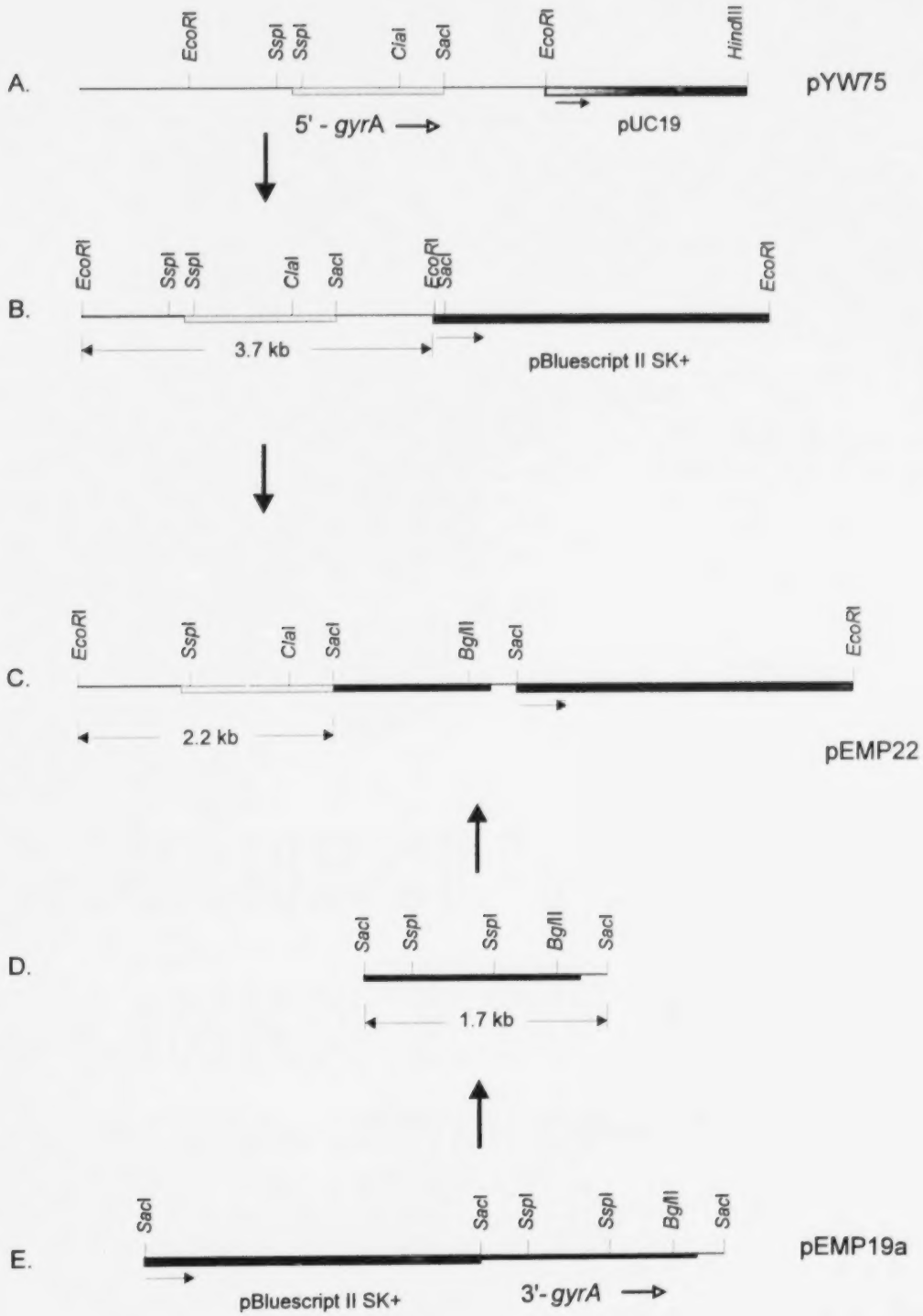
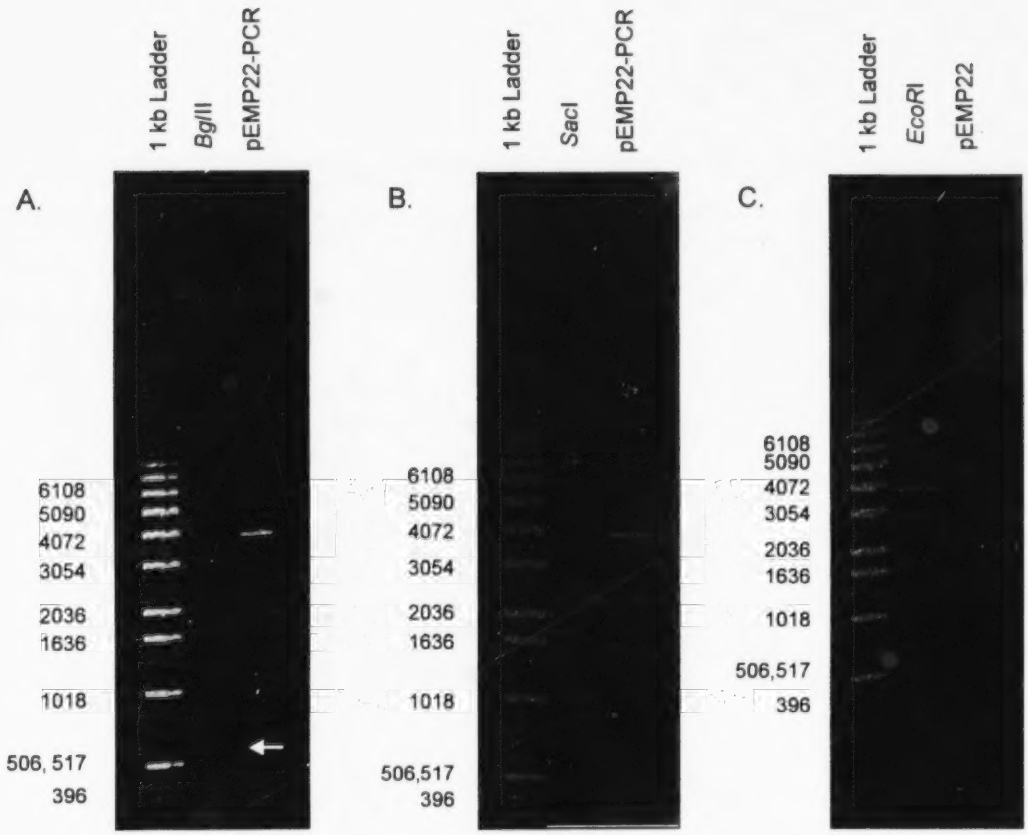

A.
EcoRI
SspI
SspI
ClaI
SacI
EcoRI
HindIII
pYW75
5' - gyrA
pUC19
B.
EcoRI
SspI
SspI
ClaI
SacI
EcoRI
SacI
EcoRI
3.7 kb
pBluescript II SK+
C.
EcoRI
SspI
ClaI
SacI
BglII
SacI
EcoRI
2.2 kb
pEMP22
D.
SacI
SspI
SspI
BglII
SacI
1.7 kb
E.
SacI
SacI
SspI
SspI
BglII
SacI
pEMP19a
pBluescript II SK+
3'- gyrA

Figure 3.12. Restriction digests of chimeric *gyrA* plasmid pEMP22.

The 4 kb PCR product yields 3.4 kb and 600 bp (white arrow) fragments (lane 2) when digested with *Bgl*II (A), and two fragments of sizes 2.3 kb and 1.7 kb (lane 2) when digested with *Sac*I (B). EcoRI digestion of the plasmid results in excision of the insert resulting in 3.9 kb and 2.9 kb fragments (lane 2) corresponding to insert and vector respectively (C). In each, uncut PCR product or plasmid is in lane 3.


A.
1 kb Ladder
BglII
pEMP22-PCR
6108
5090
4072
3054
2036
1636
1018
506, 517
396
B.
1 kb Ladder
SacI
pEMP22-PCR
6108
5090
4072
3054
2036
1636
1018
506,517
396
C.
1 kb Ladder
EcoRI
pEMP22
6108
5090
4072
3054
2036
1636
1018
506,517
396

was transformed into $CaCl_2$-competent DH10B. Transformants were screened by PCR using T3/T7 primers and a single product of about 3.9 kb was observed (Figure 3.12A, pEMP22-PCR lane.). Digestion of the PCR product with *Bgl*II and *Sac*I indicated the fragments were of the correct orientation (Figures 3.12A and 3.12B). Purified plasmid DNA was digested with *EcoR*I yielding two fragments of 3.9 kb and 2.9 kb, which correspond to insert and vector respectively (Figure 3.12C).

A kanamycin (km) resistance cassette (*aphA-3*) from *C. coli* (Trieu-Cuot *et al.*,1985) was obtained from plasmid pUOA13 (Wang, 199) as a 1.4 kb *ClaI-HindIII* fragment and ligated directly into pBluescript II SK+ cut with the same enzymes. Kanamycin-resistant transformants were selected and plasmid DNA screened for the presence of the 1.4 kb *aphA-3* insert. DNA was isolated from a positive recombinant and cut with *EcoR*I and treated with Shrimp Alkaline Phosphatase (SAP) prior to ligation with the 3.9 kb *EcoR*I fragment from pEMP22 containing the chimeric *gyrA* gene. These were also selected for on kanamycin-supplemented LB agar since treatment with SAP should reduce the background of recircularized vector. An unexpected result was obtained when recombinant plasmid DNA was isolated from these transformants for viaual analysis. Electrophoresis on 1.0% agarose gels followed by EtBr staining resulted in the appearance of a ladder of plasmid DNA in 6 out of 10 transformants, which is consistent with what would be expected to be observed with deficient gyrase function. *EcoR*I digestion of these plasmids only identified a single 4.4 kb fragment, which corresponds to the size of the cloning vector (2986 bp) plus the aphA-3 fragment (1.4 kb). This indicates that the chimeric *Campylobacter gyrA* fragment had not been successfully inserted. In addition, PCR analysis of the plasmids from these strains using T3/T7 primers to amplify the multiple cloning region yielded only fragments of 1.5 kb in size as is expected from insertion of only

the kanamycin resistance determinant. PCR analysis of crude whole DNA preparations from these strains was also negative for chimeric *gyrA* sequences when using primers specific to the *C. jejuni* and *C. fetus* regions of *gyrA*. This result suggests that there was no recombination between plasmid and chromosomal DNA.

3.8 Isolation of gyrase holoenzyme

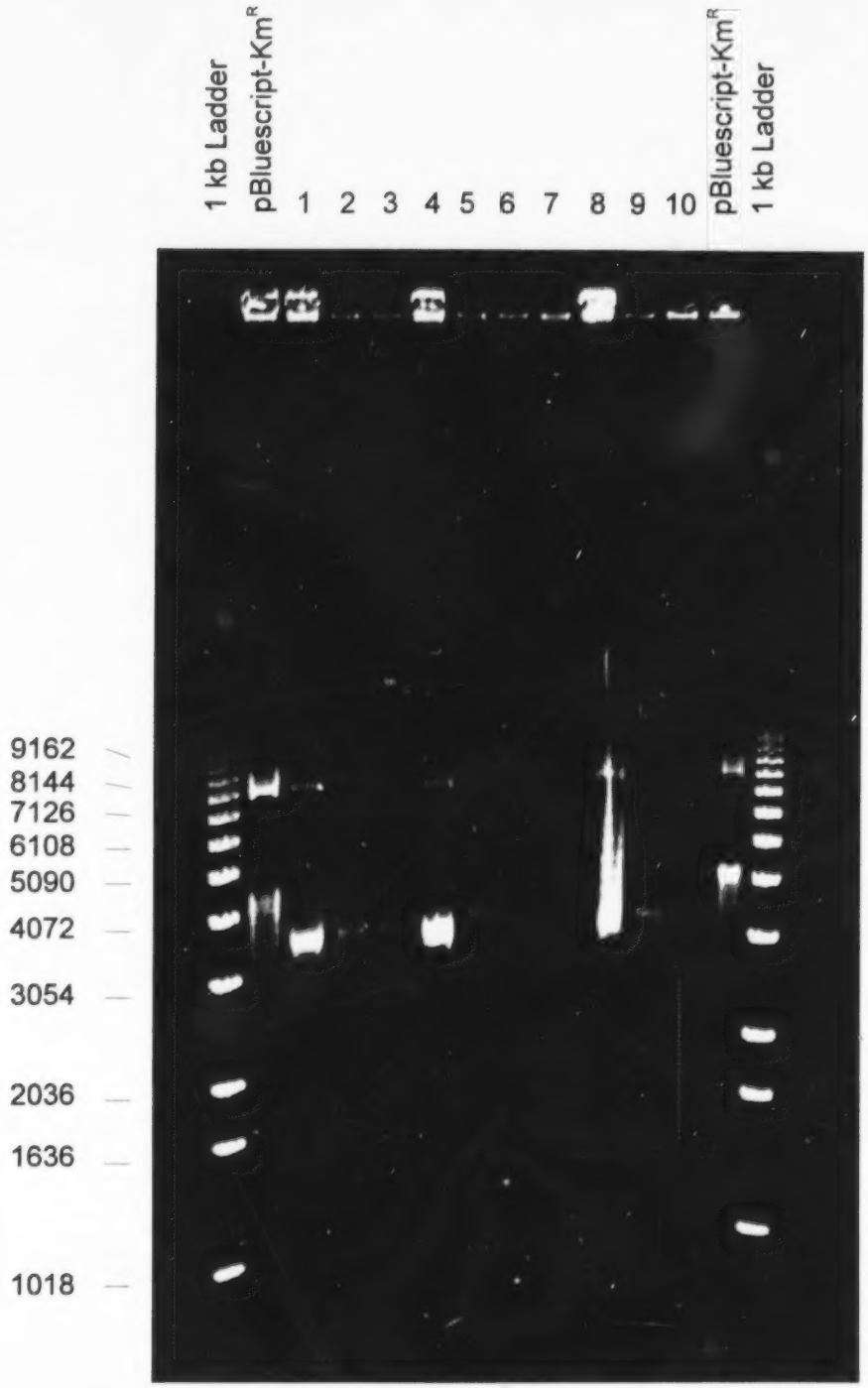
No detectable supercoiling activity was observed with gyrase protein preparations. Analysis on a Coomassie-stained non-denaturing polyacrylamide gel of the final 5M urea/HEPES fraction indicated only 5 different proteins in the samples. This is in contrast to the distribution and number of proteins in the cleared sonicated supernatant as well as that seen in each of the lanes corresponding to the heparin-agarose 0.2 M KCl/TGED eluate, or the 0.5 M KCl/TGED eluate. It was difficult to determine the protein concentration because of complicating factors: (i) interference from either glycerol or urea present in the samples; or, (ii) low concentration of final product that was undetectable with UV absorbance. Supercoiling assays were negative which suggested the protein was either not present or inactive fractions. PAGE analysis of the final fractions revealed very little protein with Coomassie blue staining although faint bands close to the expected size could be observed.

3.9 Nalidixic acid accumulation studies

The time-course studies on nalidixic acid accumulation in both *C. fetus* UA60 and *C. jejuni* UA580 indicated there was no difference the two strains. Both exhibited similar

Figure 3.13. Electrophoretic analysis of recombinant Km^R plasmids isolated from *E. coli*.

DNA from *E. coli* harboring kanamycin-resistant derivatives of pBluescript II SK+ possessing the *aphA-3* determinant are in lanes 2 and 11. Lanes 3 through 13 (labeled 1 trhough 10) are samples of crude plasmid preparations from *E. coli* cells from experiments attempting to construct chimeric *E. coli/Campyloybacter gyrA* genes using these kanamycin-resistant plasmids.


1 kb Ladder
pBluescript-KmR
1 2 3 4 5 6 7 8 9 10
pBluescript-KmR
1 kb Ladder
9162
8144
7126
6108
5090
4072
3054
2036
1636
1018

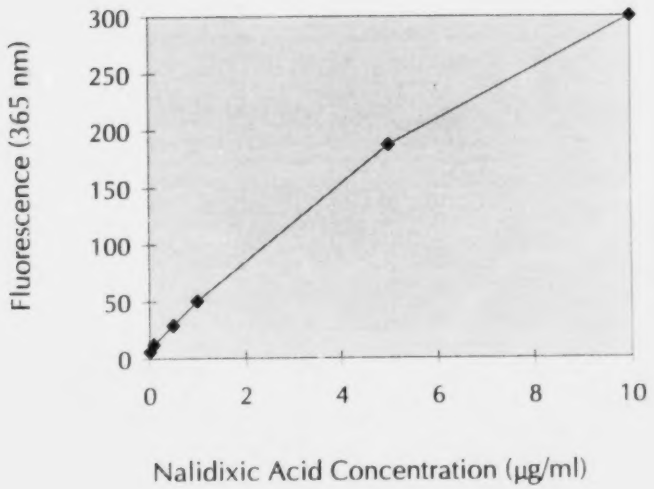
low levels of accumulation as they peaked at about 0.5 µg/ml. In addition, there was no difference in readings whether CCCP was present in the buffer or not. The UA585 data is not shown but the fluorometric readings were about one-half observed with UA60 (see Figure 3.14).

Figure 3.14. Graph of nalidixic acid accumulation by *C. fetus* UA60.

(A) Graph of standard curve determination of nalidixic acid in glycine-HCl determined using a fluoresence assay with absorption and emission values of 325 nm and 365 nm respectively. (B) Graph of nalidixic acid accumulation by *C. fetus* UA60 in the presence (triangles) and absence (squares) of the proton pump uncoupler CCCP.

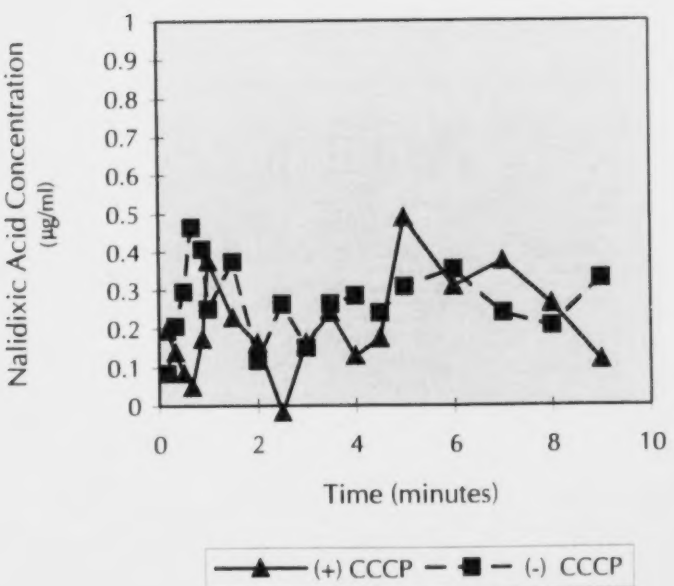
A.

Nalidixic Acid Standard Curve



B.

UA60 Nalidixic Acid Accumulation



4. Discussion

Since antibiotic drugs are such an important component of microbial medicine in therapy as well as prophylaxis, it is important to understand both their structure and function, the mechanisms by which they are effective, and their uptake by and sites of action in bacteria. A corollary to this is the idea that it is crucial to understand the molecular mechanisms of resistance to antibiotics on the part of bacteria. Bacteria have the ability to adapt to a variety of environmental stresses, partly due to the rapid appearance of mutants as a consequence of their short generation times. Although mutation frequencies may not be abnormally high, their short life cycles coupled with large population pools contribute to the ability to select for mutant traits. One aspect of this response is the appearance of antibiotic resistances. Complementing the occurrence of spontaneous mutations, resistance determinants may also be introduced via conjugation, transduction, transposon insertion or transformation (Davies, 1994). This means bacteria may acquire resistance determinants from other bacteria in nature or within hosts. As a result, there has been an alarming increase in the incidence of drug-resistant bacteria leading to more frequent treatment failures in clinical settings (Travis, 1994). With the paucity of novel antibacterial compounds, natural or synthetic, it is of even greater importance that existing resistance mechanisms be identified and characterized because only with knowledge and understanding is it possible to reverse the current trend.

Campylobacter fetus subsp. *fetus* is a well known and recognized pathogen which causes sporadic abortion in cattle and sheep. Transmission is thought to be via contaminated food or water due to feces, aborted fetuses, or vaginal discharges from aborting animals. Infection is thought to originate after ingestion and translocation to the

intestine. These bacteria have a tropism for placental tissue and during the course of bacteremia invade the uterus and mutiply in the tissue of the immature fetus. Diagnosis is by the isolation of bacteria from either the placenta or the organs of the infected fetus. Due to it's propensity for causing abortions, *C. fetus* is a major economic concern to the agricultural industry. In addition to its importance in veterinary medicine, *C. fetus* has also been shown to be a human pathogen. Although not as prevalent as *C. jejuni* diseases, those caused by *C. fetus* are often of a more serious nature; in most cases, there is some underlying medical complication that predisposes individuals to infection. Aside from the occasional case of diarrhea, the predominant feature of *C. fetus* infection is bacteremia. In contrast, in *C. jejuni* infections, diarrhea is the major feature. One explanation for this difference is the presence of an S-layer in *C. fetus* that confers serum resistance and prevents C3b binding; and it may also be the mechanism by which this organism evades host defence factors through variation of its constituent S-layer surface proteins (Blaser, 1993). These virulence characteristics are well suited for allowing these organisms to survive in the bloodstream. As a result the probability of a more serious infection caused by *C. fetus* is greater than expected with *C. jejuni*, which are serum sensitive. *C. fetus* can also cause meningitis, pericarditis, peritonitis, salpingitis, septic arthritis, and abscesses.

Normal treatment of systemic infections is with erythromycin and, occasionally, ampicillin (Blaser, 1990). For vascular infections gentamicin is the therapy of choice with ampicillin or impenem/cilastatin being alternatives. CNS infections are treated with ampicillin or chloramphenicol. Unlike *C. jejuni* or *C. coli*, which are regularly isolated from feces of infected individuals, *C. fetus* is usually identified from blood cultures. They may also be infrequently isolated from stool samples from individuals with diarrhea or bacteremia. Although neither ciprofloxacin nor nalidixic acid are significant therapeutic

agents in the treatment of *C. fetus* infections, it is important to elucidate the mechanisms responsible for the high intrinsic resistance to the latter drug. This will allow for a better understanding of bacteria-drug interactions and may shed light on possible directions of research to deal with the increasing incidence of quinolone resistance in clinical settings. With the popularity of quinolones as a supplement in the feeds of different agricultural animals, the incidence of quinolone-resistant environmental strains is increasing (Endtz *et al.*, 1991).

Comparisons of *gyrA* nucleotide and deduced amino acid sequences reveals that *C. fetus* subsp. *fetus* and *C. jejuni* proteins are closely related. The biochemical and physiological differences that exist between these two species notwithstanding, this result is not unexpected due to their apparent similarities in other aspects and classification within the same genus. Using data from 23s rRNA hybridization studies (Vandamme *et al.*, 1991) and similarity of physical characteristics as measures of relatedness, *C. fetus* and *C. jejuni* are closely related, as is *H. pylori*. Phylogenetic classification places these three species in rRNA superfamily VI; both *C. fetus* and *C. jejuni* are members of rRNA cluster I and *H. pylori* is contained in rRNA cluster III. In addition, 16S rRNA analysis places these three species in the epsilon subdivision of the Proteobacteria (Trust *et al.*, 1994). To date, 23 different complete *gyrA* sequences from various bacteria have been submitted to the GenBank nucleic acid database. Initial amino acid alignments were performed with a BLASTP search of the PIR and SWISS-PROT protein databases which reports ungapped matches only. Ten sequence files from the alignments with the highest similarity scores were retrieved and used as input for the PILEUP program of the Wisconsin GCG Software Package. Using a gap weight value of 1.0 and gap formation penalty of 0.10, complete *C. fetus*, *C. jejuni* (Wang and Taylor, 1993), *H. pylori* (Moore *et al.*, 1995), *B. subtilis* (Moriya

et al., 1985), *E. carotovora* (Rosanas, unpublished), *E. coli* (Swanberg and Wang, 1987), K. pneumoniae (Dimri and Das, 1990), *N. gonorrhoeae* (Belland et al., 1994), and *P. aeruginosa* (Margerrison *et al.*, 1992) GyrA sequences were aligned. Based on both overall nucleotide and amino acid sequence homology the *C. fetus* GyrA is most closely related to *C. jejuni* with an 78% identity between proteins and 73% homology at the nucleic acid level. Similarly, UA60 GyrA also shares a high degree of similarity with *H. pylori* for which sequence identity and DNA homology are both 63%.

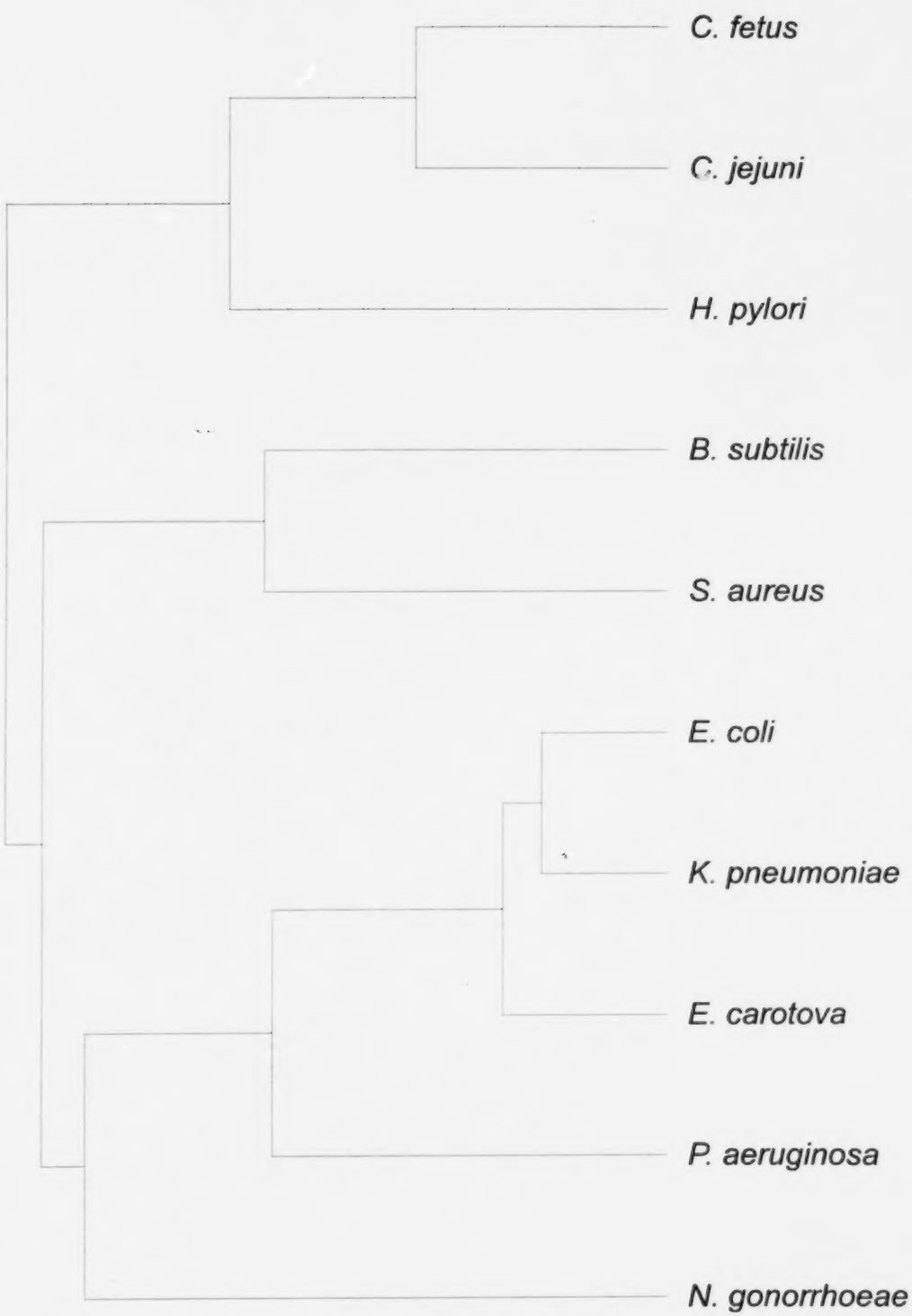
This high detree of similarity can be rationalized in one of two ways. As one of the 'housekeeping' genes, there is an absolute requirement for DNA gyrase (Reece and Maxwell, 1993). It is the only known enzyme that can introduce negative supercoils in vivo in bacteria and mutants defective for gyrase can only be rescued by the presence of wild-type copies of the genes on plasmids. At a molecular level, GyrA has to bind, cleave, and religate duplex DNA; so intuitively, there is a functional homology that may be reflected at the structural level. Theoretically, the number and combinations of amino acids that could make up a protein, thus ultimately determine its tertiary structure, are dependent only on the number of residues that make up the protein (Brändén and Tooze, 1991). This can be illustrated by the concept of proteins as an assembly of structural mofifs. For example, if the basic structure of a functionally related group of proteins is a β-barrel with α-helices at one end, any combination of amino acids that allows for this arrangement, while satisfying spatial requirements of active-site residues, could comprise the primary structure. This is because the primary factor that determines protein structure is the composition and order of the amino acid sequence. In practice, functionally related proteins that share structural features also display a degree of residue conservation within these regions, because not all side chains are compatible, therefore they cannot be

substituted randomly. As a result, there is usually an amino acid sequence motif common to functionally-related proteins or regions of proteins.

Another way to explain the alignment data is in phylogenetic terms. If gyrases were to be thought of as being divergent proteins, all arising from an ancestral precursor gyrase, it can be reasoned that the amino acid sequence of GyrA is a measure of evolutionary relatedness. This could explain the similarities between *C. fetus* and *C. jejuni* as well as *C. fetus* and *H. pylori* and *C. fetus* with other bacteria. As discussed earlier, the Campylobacters are classified together phylogenetically and *H. pylori* is grouped closer to them than either *E. coli* or *B. subtilis*. Examination of the phylogenetic representation of the alignment shows the relatedness in graphical form (Figure 4.1). The Campylobacters and *H. pylori* form their own major branch separate from the other genera. The GyrA from the Gram-positives are more closely related to these other Gram-negatives than to the Campylobacters and *H. pylori*. However, in the phylogenetic tree, they are on a separate branch from the Gram-negatives and these divisions match rRNA-based classification of these organisms. There is functional evidence to support this view in that the GyrA protein is divided into an N-terminal and C-terminal domain (Reece and Maxwell, 1991), with the DNA binding and cleavage activities mapping to the highly conserved N-terminal region and protein stablization functions associated with more divergent C-terminal fragment. What appears to be true is that both functional and structural homologies and protein evolution have led to the degree of conservation observed. There are certain amino acid sequence motifs which are absolutely conserved in 100% of the sequences aligned. An example of these is the sequence GIAVMAT. A search of the protein sequence motif database in the GCG package with the program MOTIFS found no similarity with previously characterized sequence motifs in GyrA. Wang and Taylor (1993) and Moore *et al.* (1995) found that both *C. jejuni* and *H. pylori* shared an amino acid stretch found only

Figure 4.1 Phylogenetic tree representation of the alignment of GyrA proteins.

Sequences from *C. fetus, B. subtilis, C. jejuni, E. carotova, E. coli, H. pylori, K. pneumoniae, N. gonorrhoeae, P. aeruginosa,* and *S. aureus* generated by the GCG program PILEUP. (See the legend for Figure 2.8 for a description of the sequence accession numbers and sources.)


C. fetus
C. jejuni
H. pylori
B. subtilis
S. aureus
E. coli
K. pneumoniae
E. carotova
P. aeruginosa
N. gonorrhoeae

in *P. aeruginosa* around residues 666 to 701 and 607 to 652 respectively. *C. fetus* GyrA also possesses a similar region and shares the most homology, with respect to length, with the *C. jejuni* sequence. Despite their high level of similarity, this is one region of the protein that is the most dissimilar between the two species, with little or no significant homologies at either the nucleic acid or amino acid level. In *C. fetus*, this region spans amino acid residues 658 to 691. An interesting but unexpected result from the GyrA alignment data is that *N. gonorrhoeae* is similar to these genera in that it also has this region. However, there is no sequence similarity, only the physical similarity of having a stretch of extra residues compared to *E. coli*. The obvious question that arises is whether this region has any significance. Keeping in mind the physical delineation of the protein on the basis of function, this region may encode specificity to these particular bacteria. In fact, *E. coli*, *E. carotovora*, *K. pneumoniae*, *N. gonorrhoeae* and *P. aeruginosa* also share a region, near the centre of the protein sequence not found in the other four bacteria that were previously discussed. These bacteria are more related to one another with the exception of *N. gonorrhoeae* than with the other five species which implies some role in species or genus specificity for this protein. Perhaps this region interacts with the GyrB subunit, explaining why *C. jejuni* and *H. pylori gyrA* genes were so difficult to clone or could not be expressed in *E. coli*.

Another interesting observation is that the percent amino acid identity matches the percent nucleic acid homology between *C. fetus* and *C. jejuni*. The question arises, whether these differences in the nucleic acid sequence occur at the third or 'wobble' position of each triplet codon or are they evenly distributed among the three bases? A survey of codon usage in *C. fetus*, *C. jejuni* and *H. pylori*, with the CODONFREQUENCY program of GCG, indicates that the majority of changes are silent but there are some

differences in amino acid compositions (Table 4.1). *C. fetus* appears to be intermediate relative to the other two bacteria when examining GC bias in the third position. *C. jejuni* has a preference for A or T's in the wobble position whereas *H. pylori* displays a more equal distribution of the different bases. Although both *C. fetus* and *C. jejuni* are members of the *Campylobacters,* they are not as related to one another as *C. fetus* is to *C. hyointestinalis* or *C. jejuni* is to *C. coli.* The presumption is that the nucleotide and amino acid sequences of a *C. hyointestinalis gyrA* gene should share the greatest homology with *C. fetus* as would a *C. jejuni* and *C. coli* pair. There is about a 49% overall identity for all the GyrA proteins which is extremely high, considering the spectrum of environments and backgrounds of the many different species represented. At the same time, it makes sense as DNA gyrase is an extremely important protein and functional as well as phlyogenetic influences appear to contribute to this conservancy.

It has been known that gyrase was assoicated with the nalidixic acid resistance phenotype since 1977 (Gellert *et al.*). Subsequent research revealed that the bulk of resistance-conferring mutations occurred within GyrA with a few, less significant, mutations in GyrB (Yoshida *et al.*, 1991). The association of the QRDR of *gyrA* with resistance to quinolones was first reported for *E. coli* by Yoshida and co-workers (1990) and has since been supported by other investigators studying both Gram negative and Gram positive bacteria. In those bacteria that have been studied, resistance to quinolone antibiotics correlates well with amino acid substitutions within this stretch from residues 67 to 106 in *E. coli* (reviewed in Hooper and Wolfson, 1993). For example, in *E. coli,* alteration of Ser-83 to Leu is associated with increases in resistance to quinolones and fluoroquinolones (Yoshida *et al.*, 1990). However, mutations in this region are not restricted to this residue nor these particular subsitutions. Similar substitution mutations have been reported for *C. jejuni* (Wang and Taylor, 1993), *H. influenzae* (Setlow et al.,

Table 4.1 Codon usage in gyrA genes of *Campylobacter* and *Helicobacter*.

Survey of codon usage and amino acid distribution in *C. fetus* UA60, *C. jejuni* UA580 (accession number I04566) and *H. pylori* (accession number I29481) using the GCG program CODONFREQUENCY. *C. jejuni* and *H. pylori* sequences were obtained from GenBank.

		C. fetus	*C.jejuni*	*H. pylori*			*C. fetus*	*C.jejuni*	*H. pylori*
Amino Acid	**Codon**	**Number**	**Number**	**Number**	**Amino Acid**	**Codon**	**Number**	**Number**	**Number**
G	**GGG**	2	3	16	**W**	**TGG**	1	1	0
G	**GGA**	15	15	5	**0**	**TGA**	0	0	1
G	**GGT**	25	33	10	**C**	**TGT**	3	4	1
G	**GGC**	15	3	23	**C**	**TGC**	2	0	1
E	**GAG**	25	20	18	**0**	**TAG**	0	0	0
E	**GAA**	38	62	56	**0**	**TAA**	0	1	0
D	**GAT**	65	58	43	**Y**	**TAT**	15	19	17
D	**GAC**	16	5	10	**Y**	**TAC**	3	1	6
V	**GTG**	5	12	31	**L**	**TTG**	10	14	22
V	**GTA**	26	18	3	**L**	**TTA**	29	32	36
V	**GTT**	32	26	11	**F**	**TTT**	19	21	17
V	**GTC**	7	3	14	**F**	**TTC**	6	5	7
A	**GCG**	7	3	17	**S**	**TCG**	2	0	2
A	**GCA**	17	19	6	**S**	**TCA**	5	5	2
A	**GCT**	24	24	22	**S**	**TCT**	11	13	12
A	**GCC**	5	5	10	**S**	**TCC**	1	0	2
R	**AGG**	3	3	8	**R**	**CGG**	0	0	2
R	**AGA**	20	23	16	**R**	**CGA**	2	2	2
S	**AGT**	13	24	7	**R**	**CGT**	11	17	9
S	**AGC**	25	12	25	**R**	**CGC**	11	5	12
K	**AAG**	13	9	20	**Q**	**CAG**	3	3	2
K	**AAA**	57	59	43	**Q**	**CAA**	15	19	16
N	**AAT**	32	43	25	**H**	**CAT**	8	10	10
N	**AAC**	7	4	19	**H**	**CAC**	3	1	4
M	**ATG**	26	24	22	**L**	**CTG**	2	0	2
I	**ATA**	43	21	6	**L**	**CTA**	8	2	4
I	**ATT**	15	48	46	**L**	**CTT**	29	30	12
I	**ATC**	17	13	24	**L**	**CTC**	2	1	9
T	**ACG**	7	9	6	**P**	**CCG**	4	0	1
T	**ACA**	10	16	5	**P**	**CCA**	12	10	7
T	**ACT**	18	12	13	**P**	**CCT**	7	11	11
T	**ACC**	7	8	13	**P**	**CCC**	1	0	5

1985), *H. pylori* (Moore *et al.*, 1995), *P. aeruginosa* (Kureishi *et al.*, 1994), and *S. aureus* (Fasching *et al.*, 1991; Goswitz *et al.*, 1992; Sreedharan *et al.*, 1990). In these organisms, there is an increase in MICs of quinolones and fluoroquinolones suggesting a mechanism of resistance that extends to all drugs with the basic quinolone structure. In *C. fetus*, despite the high level of nalidixic acid resistance, there are no significant differences within the QRDR between it and the other *gyrA* sequences. Compared to *C. jejuni*, there are only seven differences within this 40 amino acid long stretch and none of these appear to be non-conservative or disruptive substitutions.

This result was not completely unexpected since *C. fetus* was known to be intrinsically resistant to high levels of nalidixic acid (256 to 512 μg/ml), but unlike spontaneous *C. iejuni* or *C. coli* mutants, this resistance did not extend to fluoroquinolones (Taylor *et al.*, 1985). The MIC of ciprofloxacin for UA60 is <0.125 μg/ml, which is contrasted with the range of 1-64 μg/ml seen with nalidixic acid resistant *C. jejuni* mutants (Wang *et al.*, 1993) with MICs of nalidixic acid in the 32-128 μg/ml range. For this reason, there was no basis for suspecting that similar differences in the QRDR would be responsible for the nalidixic acid resistance profile. These data support the proposal that two mechanisms of quinolone reistance exist in Campylobacters (Taylor and Courvalin, 1988). Sequence analysis of this region from UA1221, a ciprofloxacin resistant *C. fetus* subsp. *fetus* isolate, indicates this resistance is by a mechanism analagous to that seen in other bacteria. The MIC of ciprofloxacin for UA1221 is 32 μg/ml and this increase is associated with a mutation in the QRDR to Tyr-91 from Asp. The MIC of nalidixic acid was 512 μg/ml as compared to 256 μg/ml for UA60. A laboratory mutant of UA60 that was resistant to ciprofloxacin (8 μg/ml) was also sequenced in the QRDR using a PCR

product and this supports the results from UA1221 analysis; there was only a single amino acid change at Asp-91 to a Tyr.

There are many possible explanations for a large difference in the nalidixic acid MICs: error in preparation of media for MIC determination, normal variation, or experimental error from quantiative differences in cell plating. These determinations were repeated in triplicate three times and these values were consistently obtained which would support their validity. Again, this supports the hypothesis that whichever mechanism is responsible for ciprofloxacin resistance is separate from the intrinsic resistance mechanism to nalidixic acid and in UA1221 both contribute to the overall resistance profile. However, when examining the quinolone MIC data from the literature, the high degree of variability of the values obtained for what would seem to be identical mutations tend to argue for another or other mechanisms of killing. Oram and Fisher (1991) found that equivalent Ser-83→Leu subsititutions in *E. coli* yielded MIC values for nalidixic acid ranging from 125 µg/ml to >1000 ug/ml and from 0.25 µg/ml to 1 µg/ml for ciprofloxacin. Conceptually, if GyrA is the intracellular target for quinolones and the drug's effects are manifested via altered gyrase function, only small strain-to-strain variations in MIC values would be expected. A difference of 256 µg/ml between the *C. fetuses* or 875 µg/ml between *E. coli* strains in the nalidixic acid MIC data cannot be considered trivial. There is still a possibility strain differences do exist which leads to these fluctuations in MICs as outer membrane differences, efflux pumps or other factors may contribute to the observed MIC values and cannot be discounted.

The substitution of a bulky hydrophobic residue for the smaller charged polar side group is well documented (reviewed in Hooper and Wolfson, 1993). There are three possible perturbations with this type of exhange. The substitution of a bulky nonpolar

group may introduce or increase stearic hinderance which may interfere with quinolone interaction in this region. Alternatively, the free energy of quinolone binding may be less favorable because a potential electrostatic bonding pair is eliminated or there is reduced hydrogen bonding potential. These bulky nonpolar groups may be disruptive due to repulsive interactions, eg., hydrophobic side chains tend to repel polar residues. In all likelihood it is a combination all these factors contributing to a less favorable interaction between fluoroquinolone and gyrase-DNA complex resulting in increased resistance observed in UA1221 and UA60R8.

Without structural data to support a model, there have been a number of hypothetical models. Depending on how gyrase-DNA-quinolone binding data are interpreted, the drugs may either interact directly with single stranded DNA or with the gyrase molecule or a combination of both. The latter proposal may better explain how single amino acid substitutions could drastically affect quinolone binding by eliminating bonding pairs, introducing stearic hinderance, and altering the putative binding pocket. Willmott and Maxwell (1993) found that norfloxacin and ciprofloxacin had reduced affinity *in vitro* for the *E. coli* gyrase-DNA complex when GyrA had a Ser-83→Trp mutation. This effect was not seen with either DNA or mutant DNA gyrase alone. Based on these observations it is difficult to rationalize how single point mutations in the enzyme could affect the hydrogen bonding between DNA and quinolone. This would be consistent with observations that in the presence of quinolones and protein denaturing agents, duplex DNA is cleaved leaving 4 bp staggered ends (Gellert *et al.*, 1980; Morrison and Cozzarelli, 1979). This suggests that quinolone interaction with the gyrase-DNA complex may prevent re-sealing of the cleaved ends by blocking sites on the enyzme. It is possible that quinolone binding interferes with the free 3'-hydroxyl DNA ends, thus inhibiting religation.

However, a molecular structural model of the QRDR in the presence of ciprofloxacin would have to be elucidated in order for this to be confirmed or discounted. One set of experiments that could be performed to determine whether these substitutions are significant would be to replace residues within this region in a step-wise manner by site-directed mutagenesis and then reintroduce them by homologous recombination into *C. fetus* to determine the effect on MICs of ciprofloxacin. Another direction that was pursued was to determine whether gyrase was actually responsible for nalidixic acid resistance in *C. fetus*.

It was hoped that an *in vitro* supercoiling assay using GyrA and B proteins isolated from UA60 in the presence of inhibiting concentrations of nalidixic acid would answer the question of whether gyrase was responsible for this phenotype. Isolated protein was incubated with closed-circular relaxed plasmid pBR322 but no acitive protein preparations were obtained. If the enyzme were to be resistant to the effects of nalidixic acid, a thorough examination of both GyrA and GyrB would have to be undertaken. Until this can be determined, it is difficult to speculate about the involvement of *C. fetus* GyrA in this resistance.

Other mechanisms could be active in UA60 including altered permeation at the outer membrane or the presence of an efflux pump, which actively pumps nalidixic acid back out into the environment. Resistance relating to altered outer membrane proteins, leading to decreased quinolone permeability, or efflux mechanisms typically exhibit modest increases in MICs (Cambau and Gutmann, 1993). To examine whether an efflux pump was involved with nalidixic acid resistance, UA60 and UA580 were examined for quinolone accumulation. As a preliminary investigation only, there were no significant observable differences in the fluorescence profile between *C. fetus* and *C. jejuni* either in

the presence or absence of CCCP. The difference in accumulation profiles is most likely due to experimental error. The plots were similar in overall appearance which indicates similar behavior with respect to nalidixic acid permeability. In other bacteria that have been studied, quinolone accumulation rates were rapid; plateaus were usually reached within minutes (Mortimer and Piddock, 1991). Two preliminary conclusions can be drawn from this data. The first is there does not appear to be an effux mechanims contributing to nalidixic acid resistance and the second is there does not seem to be reduced permeability of this compound in *C. fetus* as compared to *C. jejuni*. Of course, this result needs to be validated with more experiments and comparison to control studies using *E. coli* as previously described by Mortimer and Piddocck (1991).

Some difficulty was initially encountered in obtaining a complete *C. fetus gyrA* clone. This was also observed in the cloning of the *C. jejuni* gene (Wang, 1993). The C-terminal portion of the UA580 *gyrA* could only be subcloned into a low copy number plasmid pK194 with low efficiency and a complete gene could not be constructed. It was speculated that cloning and/or expression of the C-terminus was lethal in *E. coli*. However, since other bacterial *gyrA* genes have been cloned in their entirety, this would have to be something unique to the Campylobacters. Unlike the N-terminal portion of the UA60 gene, the C-terminal region proved to be more difficult to subclone. The 2.7 kb *Bgl*II fragment, corresponding to the internal portion of GyrA, or smaller-sized derivatives of this fragment could not be cloned. Fragments corresponding to this size were excised from agarose gels of chromosomal digests. Chromosomal DNA was cut with enzymes and 'shotgun' ligated into both high copy number and low copy number vectors. Restricted chromosomal DNA was gel-filtration size-selected to remove more easily ligated smaller fragments prior to ligation. Neither of these methods yielded any positive results despite there being a large

pool of recombinant plasmids of a large variety of insert sizes. These results were taken to imply that this was not just a technical problem, rather, there was some other factor that was preventing successful ligation.

Primers were designed that were specific to the 3′ end of the N-terminal clone of *gyrA* and the downstream *Bgl*II site in the C-terminal region. The last 6 residues at the 3′ terminus of the downstream oligonucleotide were 5′-AGATCT-3′. This is the same as the *Bgl*II recognition sequence. The remaining residues were complementary to the *C. jejuni* sequence downstream of the *Bgl*II site. Despite the differences in nucleotide sequence between UA60 and UA580 at the 5′ terminus and in the middle of the primer, six complementary nucleotides at the 3′ terminus was sufficient to anchor the oligonucleotide for PCR. Coupled with the upstream primer these oligonucleotides yielded a 1.5 kb fragment from UA60 chromosomal DNA which allowed identification and cloning of the remainder of the gene.

The only differences between these clones and the 2.7 kb *Bgl*II fragment are the absence of the 1.4 kb N-terminal *Bgl*II-*Sac*I fragment and the presence of the downstream 700 bp *Bgl*II-*Sac*I fragment. Most likely, there is something about the dowstream region adjacent to the *Bgl*II site or the flanking region at the 3′ terminus of the gene that stabilizes the clone. Subsequent attempts at constructing a contiguous *gyrA* were successful utilizing the high copy number vector pBluescript II SK. One explanation for the difference between *C. fetus* and *C. jejuni* in this aspect is that there is more downstream flanking DNA included in the UA60 clone. The C-terminal 1.7 kb *Sac*I fragment was ligated with *Sac*I cut pEMP9. Both orientations were obtained and transformants with a 1.5 kb *Bgl*II-*Cla*I fragment were the correct clones. Moore and co-workers (1995) could not complement *E. coli* mutants with cloned *H. pylori gyrA* plasmid-borne genes leading them

to conclude that *H. pylori* GyrA could not be expressed in *E. coli*. There may be a similar situation with the Campylobacters.

A chimeric *gyrA* gene was constructed in *E. coli* using *C. fetus* and *C. jejuni* fragments. A 3.8 kb *Eco*RI fragment from pYW75 containing the *C. jejuni* N-terminal fragment of *gyrA* was ligated to *Eco*RI-digested pBluescript II SK. The recombinant plasmid was cut with *Sac*I, removing 3' flanking DNA from the UA580 *gyrA* clone and intervening multiple-cloning site sequence from the vector. The 1.7 kb *Sac*I UA60 C-terminal *gyrA* fragment was ligated to the *Sac*I-digested plasmid. Transformants screened with PCR yielded a single insert of expected size. Digestion of this fragment with *Bgl*II and *Sac*I yielded fragments of 600 bp/3.5 kb and 1.8 kb/2.3 kb for each enzyme respectively, which are consistent with the chimeric gene being assembled in the correct orientation and reading frame. This result is in contradiction of the *C. jejuni* results and suggests either the Campylobacters are different in this respect, or the inability to subclone the C-terminal fragment in a high-copy number vector and construct a complete gene was due to an unfortunate choice of enyzmes yielding fragments containing inappropriate regions of DNA.

The observation that plasmid DNA ladders were isolated from transformants in the experiment attempting to subclone the chimeric *gyrA* is interesting. The appearance of ladders is consistent with the existence of a population of DNA where the linking number differs by one or two. This is similar to the situation where nicked relaxed DNA is covalently closed by DNA ligase; similarly, one would expect a population of topoisomers resulting in a ladder (Depew and Wang, 1975; Pulleyblank *et al.*, 1975). In this situation, the ladder appearance may have resulted either from impaired gyrase function or enhanced topoisomerase I function. An obvious and attractive possbility is that the

chimeric GyrA is interferring with the normal expression of *E. coli* GyrA or expression of this chimera is interferring with proper subunit interaction between the A and B subunits in *E. coli*. The problem with this idea is the observation that the plasmid DNA is only the expected size of a pBluescript + Km cassette construct when restricted with *EcoR*I. In addition, PCR analysis of plasmid DNA confirms this finding as only 1.5 kb fragments are amplified when 5.4 kb fragments are expected if the chimeric *gyrA* had been cloned. The ladder is not seen with pBluescript + kanamycin cassette constructs that were not involved with ligation experiments with the chimeric *gyrA* and has not been previously observed under any other conditions. The banding profile observed does not appear to be due to concatemerization of the plasmid as the differences in electrophoretic mobility are not consistent with increases in steps of 3.3 kb, which is the expected size of the vector and kanamycin cassette construct.

From the ambiguity of the data from the nucleotide analysis of *C. fetus* subsp. *fetus* it is impossible to draw any conclusions about the mechanism of nalidixic acid resistance in this microorganism. Unlike resistance to ciprofloxacin, which was similar to other GyrA proteins described in the literature, there was no analagous alterations in the quinolone resistance determining region. Preliminary data from an experiment to determine whether differences in nalidixic acid accumulation could account for the differing resistance profiles of UA60 and UA580 suggests there is no active efflux mechanism nor does there seem to be decreased permeability in *C. fetus*. There were no appreciable differences between the two species and the addition of the proton pump uncoupler CCCP had no effect. The observation that CCCP did not alter the accumulation profile is consistent with the idea of the drug being taken up through porins in an energy-independent manner (Hirai *et al.*, 1986) or via other non-energy-requiring pathways such as the 'self-promoted' uptake

suggested by Hancock (1984). In *P. aeruginosa* and *P. vulgaris*, addition of CCCP abolished quinolone accumulation leading the investigators to suggest the presence of an active efflux mechanism (Celesk and Robillard, 1989; Chamberland *et al.*, 1989; Ishii *et al.*, 1991). Additional experimentation is required to ascertain whether the accumulation data is dependable because these preliminary results exhibit fluctuation between time points and the fluorometric readings for UA580 were problematic. However, the plot of the UA580 data was similar to UA60 with the exception that the observed fluorescence was one-half achieved with UA60. It is not expected that the difference in nalidixic acid resistance can be accounted for on the basis of membrane differences alone. In bacteria where outer membrane-associated resistance has been described, whether it was due to decreased permeability or an efflux mechanism, the levels of resistance to quinolones was extemely low compared to those levels seen with alterations in GyrA (see Hooper and Wolfson, 1993, for a review). One experiment that should yield more useful data is the examination of the effect of nalidixic acid on purified *C. fetus* DNA gyrase in an in vitro supercoiling assay. The assumption is that GyrA-associated resistance would render the enzyme more resistant to the inhibitory action of the drug. Unfortunately, no active protein fractions could be isolated in the course of this thesis. However, these results support the rRNA phylogenetic classifications of the Campylobacters and *H. pylori*. Despite the high degree of similarity at the protein level, there are still some differences between *C. fetus* and *C. jejuni*. Most of these are localized to the region of the protein thought to be involved with subunit association. One line of research that was started was to construct a chimeric gyrA gene using different fragments from both *C. fetus* and *C. jejuni*. This was successful, however, whether the construct is stable in *C. fetus* or *C. jejuni* needs to be determined. In addition, it must be also be determined whether this chimeric gene was

fused together in-frame and whether it can be expressed and what effects such a hybrid would have on *in vivo* functions and resistance to quinolone antibiotics. Future research directions should concentrate on site-directed mutagenesis to determine which residues are important and at which positions. In addition to x-ray crystallographic data, this would shed light on how quinolone antibiotics interact with DNA gyrase-duplex DNA complexes and possibly reveal how best to design new analogues to circumvent the rapid increase in resistance to quinolones.

5. Bibliography

Allergerger, F., Kasten, M. J., and Anhalt, J. P., 1991. *Campylobacter fetus* subspecies *fetus* infection. Klinische Wochen-schrift. **69**:813-816.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J., 1990. Basic local alignment search tool. Journal of Molecular Biology. **215**:403-410.

Aoyama, H., Sato, K., Fujii, T., Fujimaki, K., Inoue, M., and Mitsuhashi, S., 1988. Purification of *Citrobacter freundii* DNA gyrase and inhibition by quinolones. Antimicrobial Agents and Chemotherapy. **32**:104-109.

Bedard, J., Wong, S., and Bryan, L. E., 1987. Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. Antimicrobial Agents and Chemotherapy. **31**:1348-1354.

Belland, R. J., Morrison, S. G., Ison, C., and Huang, W. M., 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Molecular Microbiology. **14**:371-380.

Birnboim, H. D., and Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. **7**:1513-1523.

Blaser, M. J., 1993. Role of the S-layer proteins of *Campylobacter fetus* in serum-resistance and antigenic variation: a model of bacterial pathogenesis. The American Journal of the Medical Sciences. *306*:325-329.

Blaser, M. J., Smith, P. F., Repine, J. E., and Joiner, K. A., 1988. Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. Journal of Clinical Investigation. **81**:1434-1444.

Blaser, M. J., Smith, P. F., Hopkins, J. A., Heinzer, I., Bryner, J. H., and Wang, W.-L. L., 1987. Pathogenesis of *Campylobacter fetus* infections: serum resistance associated with high-molecular-weight surface proteins. Journal of Infectious Diseases. **155**:696-706.

Blaser, M. J., Taylor, D. E., and Feldman, R. A., 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiological Review. **5**:157-176.

Brändén, C.-I., and Tooze, J., 1991. Chapter 16. Prediction, engineering, and design of protein structures. In: Introduction to Protein Structure. Garland Publishing Inc., New York.

Brow, M. A., 1990. Sequencing with *Taq* DNA polymerase. In: PCR Protocols a Guide to Methods and Applications. (Innis, M. A. *et al.* Ed.) Academic Press, Inc., San Diego. 189-196

Brown, P. O., and Cozzarelli, N. R., 1979. A sign inversion mechanism for enzymatic supercoiling of DNA. Science. **206**:1081-1083.

Bourguignon, G. J., Levitt, M., and Sternglanz, R., 1973. Studies on the mechanism of action of nucleic acid. Antimicrobial Agents and Chemotherapy. **4**:479-486.

Cambau, E., Bordon, F., Collatz, E., and Gutmann, L., 1993. Novel *gyr*A point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. Antimicrobial Agents and Chemotherapy. **37**:1247-1252.

Chapman, J. S., Bertasso, A., and Georgopapadakou, N. H., 1989. Fleroxacin resistance in *Escherichia coli*. Antimicrobial Agents and Chemotherapy. **33**:239-241.

Chapman, J. S., and Georgopapadakou, N. H., 1988. Routes of quinolone permeation in *Escherichia coli*. Antimicrobial Agents and Chemotherapy. **32**:438-442.

Cohen, S. P., McMurry, L. M., Hooper, D. C., Wolfson, J. S., and Levy, S. B., 1989. Cross-resistance to fluoroquinolones in multiple antibiotic resistant (Mar) *Escherichia coli* selected by tetracycline and chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrobial Agents and Chemotherapy. **33**:1318-1325.

Cohen, S. N., Chang, A. C. Y., and Hsu, L., 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. The Proceedings of the National Academy of Sciences of the United States of America.. **69**:2110-2114.

Cover, T. L., and Blaser, M. J., 1989. The pathobiology of Campylobacter infections in humans. Annual Review of Medicine. **40**:269-285.

Crick, F. H. C., 1976. Linking numbers and nucleosomes. The Proceedings of the National Academy of Sciences of the United States of America.. **73**:2639-2643.

Crick, F. H. C., Wang, J. C., and Bauer, W. R., 1979. Is DNA really a double helix? Journal of Molecular Biology. **129**:449-457.

Crumplin, G. C., Midgley, J. M., and Smith, J. T., 1980. Mechanisms of action of nalidixic acid and its congeners. Antibiotic Chemotherapy. **8**:13-35.

Cullen, M. E., Wyke, A. W., Kuroda, R., and Fisher, L. M., 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrobial Agents and Chemotherapy. **33**:886-894.

Davies, J., 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science. **264**:375-382.

Depew, R. E., and Wang, J. C., 1975. Conformational fluctuations of DNA helix. Proceedings of the National Academy of Sciences of the United States of America. **72**:4275-

Dimri, G. P., and Das, H. K., 1990. Cloning and sequence analysis of *gyrA* gene of *Klebsiella pnuemoniae*. Nucleic Acids Research. **18**:151-156.

Dreyfuss, J.M., and Midgley, J. M., 1983. Structure-activity relationships in aromatic ring fused 4-pyridones substituted at position 3. Journal of Pharmacy and Pharmacology. **35**:75P

Drlica, K., 1992. Control of bacterial DNA supercoiling. Molecular Microbiology. **6**:425-433.

Dubreuil, J. D., Logan, S. M., Cubbage, S., Ni Eidhin, E., McCubbin, W. D., Kay, C. M., Beveridge, T. J., Ferris, F. G., and Trust, T. J., 1988. Structureal and biochemical analyses of a surface array protein of *Campylobacter fetus*. Journal of Bacteriology. **170**:4165-4173.

Dubreuil, J. D., Kostryzynska, M., Austin, J. W., and Trust, T. J., 1990. Antigenic diffeneces among *Campylobacter fetus* S-layer proteins. Journal of Bacteriology. **172**:5035-5043.

Ehtesham, N. Z., and Hasnain, S. E., 1991. Direct-in-gel hybridization, without blotting, using nick-translated cloned DNA probe. Biotechniques. **11**:718-722.

Endtz, H. P., Moutoun, R. P., van der Reyden, T., Ruijs, G. J., Biever, M., van Klingeren, B., 1990. Fluoriquinolone resistance in *Campylobacter* spp. isolated from human stools and poultry products. Lancet. **335**:787.

Ezaki, T., Takeughi, N., Liu, S., Kai, A, Yamamoto, H., and Yabuughi, E., 1988. Small-scale DNA preparation for rapid genetic identification of *Campylobacter* species with radioiosotope. Microbiology and Immunology. **32**:141-150.

Farrugia, D. C., Eykyn, S. J., Smyth, E. G., 1994. *Campylobacter fetus* endocarditis: two case reports and review. Clinical Infectious Diseases. **18**:443-446.

Fasching, C. E., Tenover, F. C., Slama, T. G., Fisher, L. M., Sreedharan, S., Oram, M., Willard, K., Sinn, L. M., Gerding, D. M., and Peterson, L. R., 1991. *gyrA* mutations in ciprofloxacin-resistant, mehicilillin-resistant *Staphylococcus aureus* from Indiana, Minnesota, and Tennessee. Journal of Infectious Diseases. **164**:976-979.

Feinberg, A. P., and Vogelstein, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry. **132**:6-13.

Fuller, F. B., 1971. The writhing number of a space curve. The Proceedings of the National Academy of Sciences of the United States of America.. **68**:815-819.

Gellert, M., Mizuuchi, K., O'Dea, M. H., and Nash, H.A., 1976a. DNA gyrase: an enzyme that introduces superhelical turns into DNA. The Proceedings of the National Academy of Sciences, U.S.A. **73**:3872-3876.

Gellert, M., O'Dea, M. H., Itoh, T., and Tomizawa, J., 1976b. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. The Proceedings of the National Academy of Sciences of the United States of America.. **73**:4474-4478.

Gellert, M., Fisher, L. M., and O'Dea, M. H., 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. The Proceedings of the National Academy of Sciences of the United States of America. **74**:4772-4776.

Gellert, M., Fisher, L. M., Ohmori, H., O'Dea, M. H., and Mizuuchi, K., 1980. DNA gyrase: site-specific interactions and transient double-strand breakage of DNA. Cold Spring Harbor Symposia on Quantitative Biology. **45**:391-

Gellert, M., 1981. DNA topoisomerases. Annual Review of Biochemistry. **50**:879-910.

Genetics Computer Group, 1991. Program manual for the GCG package, Version 7. Genetics Computer Group, Madison, Wisconsin.

Goldman, R. A., Hasan, T., Hall, C. C., Strycharz, W. A., and Cooperman, B. S., 1983. Photoincorporation of tetracycline into *Escherichia coli* ribosomes. Identification of the major proteins photolabelled by native tetracycline and tetracycline photoproducts and implications for the inhibitory action of tetracycline on protein synthesis. Biochemistry. **22**:359-368.

Gootz, T. D., Martin, B. A., 1991. Characterization of high-level quinolone resistance in *Campylobacter jejuni*. Antimicrobial Agents and Chemotherapy. **35**:840-845.

Goswitz, J. J., Willard, K. E., Fasching, C. E., and Peterson, L. R., 1992. Detection of *gyrA* gene mutations associated with ciprofloxacin resistance in methicillin-resistant *Stahpylococcus aureus*: analysis by plymerase chain reaction and automated direct DNA sequencing. Antimicrobial Agents and Chemotherapy. **36**:1166-1169.

Griffiths, P. L., 1993. Morphological changes of *Campylobacter jejuni* growing in liquid culture. Letters in Applied Microbiology. **17**:152-155.

Grollier, G., Burucoa, C., Ricco, J. B., Pezennec, L., and Fauchere, J. L., 1993. Isolation and Immunogenicity of *Campylobacter fetus* subsp. *fetus* from an abdominal aortic aneurysm. European Journal of Clinical Microbiology and Infectious Diseases. **12**:847-849.

Gurrent, R. L., Lahita, R. G., Washington, C. W., and Roberts, R. B., 1978. Campylobacteriosis in man: pathogenic mechanisms and review of 91 bloodstream infections. American Journal of Medicine. **65**:584-591.

Hallet, P. and Maxwell, A., 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of mutant proteins. Antimicrobial Agents and Chemotherapy. **35**:335-340.

Hancock, R., 1984. Alterations in outer membrane permeability. Annual Review of Microbiology. **38**:237-264.

Harvey, S. M., and Greenwood, J. R., 1983. Probable *Campylobacter fetus* subsp. *fetus* gastroenteritis. Journal of Clinical Microbiology. **18**:1278-1279.

Henikoff, S., 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene. **28**:351-359.

Higgins, N. P., Peebles, C. L., Sugino, A., and Cozzaerlli, N. R., 1978. Purifiication of subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. Proceedings of the National Academy of Sciences of the United States of America. **75**:1773-1777.

Hiraga, S., 1990. Partioning of nucleoids. Research in Microbiology. **141**:50-56.

Hirai, K., Aoyama, H., Irikura, T., Iyobe, S., and Mitsuhashi, S., 1986a. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrobial Agents and Chemotherapy. **29**:535-538.

Hirai, K., Aoyama, H., Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S., 1986b. Isolation and characterization of norfloxacin-resistant *Escherichia coli* K12. Antimicrobial Agents and Chemotherapy. **30**:248-253.

Hirai, K., Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S., 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. **31**:582-586.

Hooper, D. C., Wofson, J. S., McHugh, G. L., Winters, M. B., and Swartz, M. N., 1982. Effect of novobiocin, coumermycinA1, chlorobiocin, and their analogs on *Escherichia coli* DNA gyrase and bacterial growth. Antimicrobial Agents and Chemotherapy. **22**:662-671.

Hooper, D. C., Wolfson, J., Souza, K., Tung, C., McHugh, G., and Swartz, M., 1986. Genetic and biochemical characterization of norfloxacin resistance in *Eshcerichia coli*. Antimicrobial Agents and Chemotherapy. **29**:639-644.

Hooper, D. C., and Wolfson, J. S., 1991. The quinolones: mode of action and bacterial resistance. In: Antibiotics in Laboratory Medicine, 3rd edition. (Lorian, V., Ed.). The Williams and Wilkins Co., Baltimore. 665-690

Hooper, D. C., and Wolfson, J. S., 1993. Mechanisms of quinolone action and bacterial killing. In: Quinolone Antibacterial Agents, 2nd edition. (Hooper, D. C. and Wolfson, J. S., Ed.). American Society for Microbiology, Washington. 53-75.

Hooper, D. C., and Wolfson, J. S., 1993. Mechanisms of bacterial resistance to quinolones. In: Quinolone Antibacterial Agents, 2nd edition. (Hooper, D. C. and Wolfson, J. S., Ed.). American Society for Microbiology, Washington. 97-118.

Horowitz, D. S., and Wang, J. C. 1984. Torsional rigidity of DNA and length depence of the free energy of DNA supercoiling. Journal of Molecular Biology. **173**:75-91.

Horowitz, D. S., and Wang, J. C., 1987. Mapping the active site tyrosine of Escherichia coli DNA gyrase. Journal of Biological Chemistry. **262**:5339-5344.

Innis, M. A., and Gelfand, D. H., 1990. Optimization of PCRs. In: PCR Protocols a Guide to Methods and Applications. (Innis, M. A. *et al.* Ed.) Academic Press, Inc., San Diego. 3-12.

Inoue, Y., Sato, K., Fujii, T., Hirai, K., Inoue, M., Iyobe, S., and Mitsuhashi, S., 1987. Some properties of subunits of DNA gyrase from *Pseudomonas aeruginosa* PAO1 and its nalidixic acid resistant mutant. Journal of Bacteriology. **169**:2322-2325.

Iyobe, S., Hirai, D., and Hashimoto, H., 1991. Drug resistance of *Pseudomonas aeruginosa* with special reference to new quinolones. Antibiotic Chemotherapy (Basel). **44**:202-214.

Jobling, M. G., and Holmes, R. K., 1990. Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacZ*alpha and pUC18 or pUC19 multiple cloning sites. Nucleic Acids Research. **18**:5315-5316.

Keller, W., and Wendel, I., 1974. Stepwise relaxation of supercoiled SV40 DNA. Cold Spring Harbor Symposium on Quantitative Biology. **39**:199-

Klevan, L., and Wang, J. C., 1980. DNA gyrase-DNA complex containing 140 bp of DNA and an $\alpha_2\beta_2$ protein core. Biochemistry. **19**:5229-5234.

Koenen, M., 1989. Recovery of DNA from agarose gels using liquid nitrogen. Trends in Genetics. **5**:137.

Koval, S. F., and Murray, R. G. E., 1984. The isolation of surface array proteins from bacteria. Canadian Journal of Biochemistry and Cell Biology. **62**:1181-1189.

Kreuzer, K. N., and Cozzarelli, N. R., 1979. *Escherichia coli* mutants thermosensitive for DNA gyrase subunit A: effects on DNA replication, transcription, and bacteriophage growth. Journal of Bacteriology. **140**:425-435.

Kreuzer, K. N., and Cozzarelli, N. R., 1980. Formation and resolution of DNA catenanes by DNA gyrase. Cell. **20**:245-254.

Kureishi, A., Diver, J. M., Beckthold, B., Schollardt, T., and Bryan, L. E., 1994. Cloning and nucleotide sequnce of *Pseudomonas aeruginosa* DNA gyase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrobial Agents and Chemotherapy. **38**:1944-1952.

Lastovica, A. J., and Penner, J. L., 1983. Serotypes of *Campylobacter jejuni* and *Campylobacter coli* in bacteremic, hospitalized children. Journal of Infectious Diseases. **147**:592-

Lesher, G. Y., Forelich, E. D., Gruet, M. D., Bailey, J. H., and Brundage, R. P., 1962. 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. Journal of Medical Pharmaceutical Chemistry. **5**:1063-1068.

Lewin, C. S., Amyes, S. G. B., and Smith, J. T., 1989. Bactericidal activity of enoxacin and lomefloxacin against *Escherichia coli* KL16. European Journal of Clinical Microbiology and Infectious Diseases. **8**:731-733.

Lewin, C. s., and Amyes, S. G. B., 1990. Conditions required for the bactericidal activity of pefloxacin and fleroxacin against *Escherichia coli* KL16. Journal of Medical Microbiology. **32**:83-86.

Manavathu, E. K., Hiratsuka, K., and Taylor, D. E., 1988. Nucleotide sequence analysis and expression of a tetracycline resistance gene from *Campylobacter jejuni*. Gene. **62**:17-26.

Manavathu, E. K., Fernandez, C. L., Cooperman, B. S., and Taylor, D. E., 1990. Molecular studies on the mechanism of tetracycline resistance mediated by Tet(O). Antimicrobial Agents and Chemotherapy. **34**:71-77.

Margerrison, E. E. C., Hopewell, R., and Fisher, L. M., 1992. Nucleotide sequence of the *Staphylococcus aureus gyrB-gyrA* locus encoding the DNA gyrase A and B proteins. Journal of Bacteriology. **174**:1596-1603.

Martin, N. L., and Beveridge, T. J., 1986. Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. Antimicrobial Agents and Chemotherapy. **29**:1079-1087.

Masecar, B. L., Celesk, R. A., and Robillard, N. J., 1990. Analysis of acquired ciprofloxacin resistance in a clinical strain of *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. **34**:281-286.

Masecar, B. L., and Robillard, N. J., 1991. Spontaneous quinolone resistance in *Serratia marcescens* due to a mutation in *gyrA*. Antimicrobial Agents and Chemotherapy. **35**:898-902.

McCabe, P. C., 1990. Production of single-stranded DNA by asymmetric PCR. In: PCR Protocols a Guide to Methods and Applications. (Innis, M. A. *et al.* Ed.). Academic Press, Inc., San Diego. 76-83

McCaffrey, C., Bertasso, A., Pace, J., and Georgopapadakou, N. H., 1992. Quinolone accumulation in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. **36**:1601-1605.

McCoy, E. C., Doyle, D., Burda, K., Corbeil, L. B., and Winter, A. J., 1975. Superficial antigens of *Campylobacter* (*Vibrio*) *fetus*: characterization of an antiphagocytic component. Infection and Immunity. **11**:517-525.

McCoy, E. C., Wiltberger, H. A., and Winter, A. J., 1976. Major outer membrane protein of *Campylobacter fetus*: physical and immunological characterization. Infection and Immunity. **13**:1258-1265.

McFaydean, J. and Stockman, S., 1913. Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to enquire into epizootic abortion. Part III. Her Majesty's Stationary Office, London.

Menzel, R., and Gellert, M., 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of supercoiling. Cell. **24**:105-13.

Miller, R. V., and Scurlock, T. R., 1983. DNA gyrase (topoisomerase II) from *Pseudomonas aeruginosa*. Biochemical and Biophysical Research Communications. **110**:694-700.

Mizuuchi, K., O'Dea, M. H., and Gellert, M., 1978. DNA gyrase: subunit structure and ATPase activity of the purified enzyme. The Proceedings of the National Academy of Sciences of the United States of America. **75**:5960-5963.

Mizuuchi, K., Fisher, L. M., O'Dea, M. H., and Gellert, M., 1980. DNA gyrase action involves the introduction of transient double-stranded breaks in DNA. The Proceedings of the National Academy of Sciences of the United States of America.. **77**:1847-1851.

Mizuuchi, K., Mizuuchi, M., O'Dea, M. H., and Gellert, M., 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. Journal of Biological Chemistry. **259**:9199-9201.

Moore, R. A., Beckthold, B., Wong, S., Kureishi, A., and Bryan, L. E., 1995. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. Antimicrobial Agents and Chemotherapy. **39**:107-111.

Moriya, S., Nakai, S., and Yoshikawa, H., 1985. Structure and function of the region of the replication origin of the *Bacillus subtilis* chromosome. III. Nucleotide sequence of some 10,000 base pairs in the origin region. Nucleic Acids Research. **13**:2251-2265.

Morrison, A., and Cozzarelli, N. R., 1979. Site-specific cleavage of DNA by *E. coli* DNA gyrase. Cell. **17**:175-184.

Mortimer, P. G. S., and Piddock, J. V., 1991. A comparison of methods used for measuring the accumulation of quinolones by Enterobacteriaceae, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy. **28**:639-653.

Myers, L. L., 1971. Purification and partial characterization of a *Vibrio fetus* immunogen. Infection and Immunity. **3**:562-566.

Nakamura, S., Nakamura, M., Kojima, T., and Yosida, H., 1989. g*yrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrobial Agents and Chemotherapy. **33**:254-255.

Nakanishi, N., Yoshida, S., Wakebe, H., Inoue, M., and Mitsuhashi, S., 1991. Mechanisms of clinical resistance to fluoroquinolones in *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy. **35**:1053-1059.

Ng, L.-K., Sherburne, R., Taylor, D. E., and Stiles, M. E., 1985. Morphological forms and viability of Campylobacter species studied by electron microscopy. Journal of Bacteriology. **164**:338-343.

Okuda, J., Okamoto, S., Takahata, M., and Nishino, T., 1991. Inhibitory effects of ciprofloxacin fluoroquinolone-resistant strains of methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. **35**:2288-2293.

Oram, M., and Fisher, L. M., 1991. 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrobial Agents and Chemotherapy. **35**:387-389.

Orr, E., and Staudenbauer, W. L., 1981. An *Escherichia coli* mutant thermosensitive in the B subunit of DNA gyrase: effect on the structure and replication of the Colicin E1 plasmid in vitro. Molecular and General Genetics. **181**:52-56.

Palumbo, M., Gatto, B., Zagotto, G., and Palù, G., 1993. On the mechanism of action of quinolone drugs. Trends in Microbiology. **1**:232-235

Peck, L. J., and Wang, J. C., 1981. Sequence dependence of the helical repeat of DNA in solution. Nature. **292**:375-378.

Pei, Z., Ellison, R. T., Lewis, R. V., and Blaser, M. J., 1988. Purification and characterization of a family of high molecular weight surface-array proteins from *Campylobacter fetus*. Journal of Biological Chemistry. **263**:6416-6420.

Penner, J. L., 1988. The genus *Campylobacter*: a decade of progress. Clinical Microbiology Reviews. **1**:157-172.

Peterson, L. R., 1993. Quinolone resistance in clinical practice: occurrence and importance. In: Quinolone Antimicrobial Agents, 2nd edition (Hooper, D. C., and Wilson, J. S., ed). American Society for Microbiology, Washington. 119-137.

Pulleyblank, D. E., Shure, M., Tang, D., Vinograd, J., and Vosberg, H.-P., 1975. Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: formation of a Boltzmann distribution of topological isomers. Proceedings of the National Academy of Sciences of the United States of America. **72**:4280-4284.

Rádl, S., 1990. Sructure-activity relationships in DNA gyrase inhibitors. Pharmacology and Therapy. **48**:1-17.

Reece, R. J., and Maxwell, A., 1991. DNA gyrase: Structure and function. Critical Reviews in Biochemistry and Molecular Biology. **26**:335-375.

Rennie, R. P., Strong, D., Taylor, D. E., Salama, S. M., Davidson, C., and Tabor, H., 1994. *Campylobacter fetus* diarrhea in a hutterite colony: epidemiological observations and typing of the causative organism. Journal of Clinical Microbiology. **32**:721-724.

Rettig, P. J., 1979. Campylobacter infections in human beings. Journal of Pediatrics. **94**:855-864.

Rhodes, D. and Klug, A., 1980. Helical periodicity of DNA determined by enzyme digestion. Nature. **286**:573-578.

Robillard, N. J., and Scarpa, A. L., 1988. Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. Antimicrobial Agents and Chemotherapy. **32**:535-539.

Ronald, A. R., Norrby, S. R., and Svard, R., 1966. A critical evaluation of nalidixic acid in urinary-tract infections. New England Journal of Medicine. **275**:1081-1089.

Rosanas, A., 1994. Unpublished results.

Sagara, K., Mochizuki, A., Okamura, N., and Nakaya, R., 1987. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* with special reference to plasmid profiles of Japanese clinical isolates. Antimicrobial Agents and Chemotherapy. **31**:713-719.

Sambrook, J., Fritsch, E. F., and Maniatus, T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Samuelson, J. D., and Winter, J. A., 1966. Bovine vibriosis: the nature of the carrier state in the bull. Journal of Infectious Diseases. **116**:581-592.

Sanger, F., Nicklen, S., and Coulson, A. R., 1977. DNA sequencing with chain-terminating inhibitors. The Proceedings of the National Academy of Sciences of the United States of America. **74**:5463-5467.

Schaechter, M., 1990. The bacterial equivalent of mitosis. *In*: The Bacterial Chromosome. (Drlica, K., and Riley, M., Ed.). American Society for Microbiology Publications, Washington. 313-

Schuler, G. D., Altschul, S. F., and Lipman, D. J., 1991. A workbench for multiple alignment construction analysis. Proteins Structure Function Genetics. **9**:180-190

Schurig, G. D., Hall, C. E., Corbeil, L. B., Duncan, J. R., and Winter, A. J., 1975. Bovine venereal vibriosis: cure of genital infection in females by systemic immunization. Infection and Immunity. **11**:245-251.

Schurig, G. D., Hall, C. E., Burda, K., Corbeil, L. B., Duncan, J. R., and Winter, A. J., 1978. Elimination of genital vibriosis in female cattle by systemic immunization with killed cells or cell-free extracts of *Campylobacter fetus*. Journal of Infectious Diseases. **138**:463-472.

Segreti, J., Gootz, T. D., Goodman, L. J., Parkhurst, G. W., Quinn, J. P., Martin, B. A., and Trenholme, G. M., 1992. High-level quinolone resistance in clinical isolates of *Campylobacter jejuni*. The Journal of Infectious Diseases. **165**:667-670.

Seidman, C. E., 1987. Unit 1.8. Introduction of plasmid DNA into cells. In: Current Protocols in Molecular Biology. (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.). Wiley Interscience, New York. 1.8.1-1.8.3.

Setlow, J. K., Cabrera-Juarez, K., Albritton, W. L., Spikes, D., and Muschler, A., 1985. Mutations affecting gyrase in *Haemophilus influenze*. Journal of Bacteriology. **164**:525-534.

Shen, L. L., and Pernet, A. G., 1985. Mechanism of inhibition of DNA gyrase by anologues of nalidixic acid: the target of drugs is DNA. Proceedings of the National Academy of Sciences of the United States of America. **82**:307-311.

Shen, L. L., Kohlbrenner, W. E., Weigl, D., and Baranowski, J., 1989a. Mechanism of quinolone inhibition of DNA gyrase. Journal of Biological Chemistry. **264**:2973-2978.

Shen, L. L., Baranowski, J., and Pernet, G., 1989b. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: specificity and cooperativity of drug binding to DNA. Biochemistry. **28**:3879-3885.

Shen, L. L., Mitscher, L. A., Sharma, P. N., O'Donnell, T. J., Chu, D. W. T., Cooper, C. S., Rosen, T., and Pernet, A. G., 1989c. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. Biochemistry. **28**:3886-3894.

Shen, L. L., Kohlbrenner, W. E., Weigl, D., and Baranowski, J., 1989d. Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. Journal of Biological Chemistry. **264**:2973-2978.

Shen, L., 1993. Quinolone-DNA interaction. In: Quinolone Antimicrobial Agents. (Hooper, D. C., and Wolfson, J. S., ed). American Society for Microbiology, Washington. 77-95.

Shore, D. and Baldwin, R. L., 1983. Energetics of DNA twisting. I. Relation between twist and cyclization probability. Journal of Molecular Biology. **170**:957-981.

Skirrow, M. B., Jones, D. M., Sutcliffe, E., and Benjamin, J., 1993. *Campylobacter* bacteraemia in England and Wales, 1981-91. Epidemiology and Infection. **110**:567-573.

Sleytr, U. B., and Messner, P., 1988. Crystalline surface layers in procaryotes. Journal of Bacteriology. **170**:2891-2897.

Smibert, R. M., 1978. The genus Campylobacter. Annual Review of Microbiology. **32**:673-709.

Smit, J., 1986. Protein surface layers of bacteria. *In*: Bacterial outer membranes as model systems (Inouye, M., Ed). John Wiley and Sons, Inc., New York. 343-376.

Smith, G. R., 1981. DNA supercoiling: another level for regulating gene expression. Cell. **24**:599-600.

Smith, J. T., 1984. Awakening the slumbering potential of the 4-quinolone antibacterials. Pharmaceutical Journal. **233**:299-305.

Smith, J. T., 1986. The mode of action of 4-quinolones and possible mechanism of resistance. Journal of Antimicrobial Chemotherapy. **18**(Suppelement D):21-29.

Sougakoff, W., Papadopoulou, B., Nordmann, P., and Courvalin, P., 1987. Nucleotide sequence and distribution of gene *tetO* encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiology Letters. **44**:153-159.

Southern, E. M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology. **98**:503-517.

Spelman, D. W., Davidson, N., Buckmaster, N. D., Spicer, W. J., and Ryan, P., 1986. Campylobacter bacteraemia: a report of 10 cases. Medical Journal of Australia. **145**:503-505.

Sreedharan, S., Oram, M., Jensen, B., Peterson, L. R., and Fisher, L. M., 1990. DNA gyrase *gyrA* mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: close similarity with quinolone reistance mutation s in *Escherichia coli*. Journal of Bacteriology. **172**:7260-7262.

Staudenbauer, W. L., and Orr, E., 1981. DNA gyrase: affinity chromatography on novobiocin-Sepharose and catalytic properties. Nucleic Acids Research. **9**:3589-3603.

Steck, T. R., and Drlica, K., 1984. Bacterial chromosome segregation: evidence for DNA gyrase involvement in decatenation. Cell. **36**:1081-1088.

Steers, E., Foltz, E. L., Gravis, B. S., and Riden, J., 1955. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiotic Chemotherapy. **9**:307-311.

Sugino, A., Peebles, C. L., Kruezer, K. N., and Cozzarelli, N. R., 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. The Proceedings of the National Academy of Sciences of the United States of America.. **74**:4767-4771.

Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L., and Cozzerelli, N. R., 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. The Proceedings of the National Academy of Sciences of the United States of America.. **75**:4838-4842.

Sugino, A., and Bott, K. F., 1980. *Bacillus subtilis* deoxyribonucleic acid gyrase. Journal of Bacteriology. **141**:1331-1339.

Sugino, A., Higgins, N. P., and Cozzerelli, N. R., 1980. DNA gyrase subunit stoichiometry and the covalent attachment of subunit A to DNA during DNA cleavage. Nucleic Acids Research. **8**:3865

Swanberg, S. L., and Wang, J. C., 1987. Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. Journal of Molecular Biology. **197**:729-736.

Tauxe, R. V., Hargrett-Bean, H., Patton, C. M., and Wachsmuth, I. K., 1988. *Campylobacter* isolates in the United States, 1982-1986. Morbitiy and Mortality Weekly Report. **37**:1-13.

Taylor, D. E., De Grandis, S. A., Karmali, M. A., and Fleming, P. C., 1980. Letter. Lancet. **ii**:797.

Taylor, D. E., Ng, L.-K., and Lior, H., 1985. Susceptibility of Campylobacter species to nalidixic acid, enoxacin, and other DNA gyrase inhibitors. Antimicrobial Agents and Chemotherapy. **28**:708-710.

Taylor, D. E., Hiratsuka, K., Ray, H., and Manavathu, E. K., 1987. Characterization and expression of a cloned tetracycline resistance determinant from *Campylobacter jejuni* plamsmid pUA466. Journal of Bacteriology. **169**:2984-2989.

Taylor, D. E., and Courvalin, P., 1988. Mechanisms of antibiotic resistance in *Campylobacter* species. Antimicrobial Agents and Chemotherapy. **32**:1107-1112.

Taylor, D. E., 1992. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* to tetracycline, chloramphenicol, and erythromycin. *In*: *Campylobacter jejuni*: Current status and future trends (Nachamkin, I., Blaser, M. J., Tompkins, L. S., Ed.). American Society for Microbiology, Washington. 74-86.

Tenover, F. C., Baker, C. N., Fennell, C. L., Ryan, C. I., 1992. Antimicrobial resistance in *Campylobacter* species. *In*: *Campylobacter jejuni*: Current status and future trends (Nachamkin, I., Blaser, M. J., Tompkins, L. S., Ed.). American Society for Microbiology, Washington. 66-73.

Travis, J., 1994. Reviving the antibiotic miracle? Science. **264**:360-361.

Trieu-Cuot, P., Gerbaud, G., Lambert, T., and Courvalin, P., 1985. In vivo transfer of genetic information between Gram-positive and Gram-negative bacteria. EMBO Journal. **4**:3583-3587.

Trust, T. J., Logan, S. M., Gustafson, C. E., Romaniuk, P. J., Kim, N. W., Chan, V. L., Ragan, M. A., Guerry, P., and Gutell, R. R., 1994. Phylogenetic and molecular characterization of a 23S rRNA gene positions the genus *Campylobacter* in the epsilon subdivision of

the Proteobacteria and shows that the presence of transcribed spacers is common in *Campylobacter* spp. Journal of Bacteriology. **176**:4597-4609.

Ullmann, U., Lamgmaack, H., and Blasius, C., 1982. Campylobacteriosis des Menschen durch die Subspezies intestinalis und fetus unter Berücksichtigung sechs neuer Erkarankungen. Infection. **10** (Supplement 2):564-566.

United States Biochemical Corporation, 1991. Molecular Biology Reagents/Protocols. United States Biochemical Corporation, Cleveland, Ohio.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R., and De Ley, J., 1991. Revision of *Campylobacter, Helicobacter,* and *Wolinella* taxonomy: Emendation of generic descriptions and proposal of *Arcobacter* gen. nov. International Journal of Systematic Bacteriology. **41**:88-103.

Vaara, M., and Vaara, T., 1983. Sensitization of gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. Nature (London). **30**:526-528.

Vieira, J., and Messing, J., 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene. **19**:259-268.

Vinzent, R., Dumas, J., and Picard, N., 1947. Septicemie grave au cours de la grossesse due a un Vibrion. Avortement consecutif. Bulletin de l'Academie Nationale Medicine. **131**:90-

Wang, J. C., 1985. DNA toposiomerases. Annual Review of Biochemistry. **54**:665-697.

Wang, Y., W. H. Huang, and D. E. Taylor, 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyr*A gene and characterization of quinolone resistance mutations. Antimicrobial Agents and Chemotherapy. **37**: 457-463.

Wang, Y., and Taylor, D. E., 1990. Chloramphenicol resistance in Campylobacter coli: nucleotide sequence, expression, and cloning vector construction. Gene. **94**:23-28.

Wang, Y., 1990. Double-stranded DNA sequencing with T7 polymerase. Biotechniques. **6**:843-845.

Watson, J. D., and Crick, F. H. C., 1953. Molecular structure of nucleic acid. A structure for deoxyribonucleic acid. Nature. **171**:737-738.

White, J. H., Cozzarelli, N. R., and Bauer, W. R., 1988. Helical repeat and linking number of surface wapped DNA. Science. **241**:323-327.

White, J. H., 1969. Self-linking and the Gauss integral in higher dimensions. American Journal of Mathematics. **91**:693-

Willmott, C. J. R. and A. Maxwell, 1993. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrobial Agents and Chemotherapy. **37**:126-127.

Wyatt, R. A., Younoszai, K., Anuras, S., and Myers, M. G., 1977. *Campylobacter fetus* septicemia and hepatitis in a child with agammaglobulinemia. Journal of Pediatrics. **91**:441-442.

Yamagishi, J-L., Furutani, Y., Inoue, S., Ohue, T., Nakamura, s., and Shimizu, M., 1981. New nalidixic acid reistance mutations related to DNA gyrase activity. Journal of Bacteriology. **148**:450-458.

Yamagishi, J., Yoshida, H., Nakamura, M., and Nakamura, S., 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Molecular and General Genetics. **204**:367-373.

Yan, W., and Taylor, D. E., 1991. Sizing and mapping of the genome of *Campylobacter coli* strain UA417R using pulsed-field gel electrophoresis. Gene. **101**:117-120.

Yoshida, H., Kojima, T., Yamagishi, J., and Nakamura, S., 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. Molecular and General Genetics. **211**:1-7.

Yoshida, H., Bogaki, M., Nakamura, M., and Nakamura, S., 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Esherichia coli*. Antimicrobial Agents and Chemotherapy. **34**:1271-1272.